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Research article

Iron chelating, antioxidant, and anti-inflammatory properties of brazilin from *Caesalpinia sappan* Linn

Somjintana Taveepanich [a], Kampanart Chayajarus [a], Jutharat Jittimanee [b], Naruedon Phusri [c], Paptawan Thongdee [c], Khemmisara Sawatdee [c], Pharit Kamsri [d], Auradee Punkvang [d], Khomson Suttisintong [e], Pornpan Pungpo [a], Wanwisa Suwannaloet [b], Ruttiya Thongrung [b], Kanjana Pangjit [b,*]

[a] *Department of Chemistry and Center of Excellence for Innovation in Chemistry, Faculty of Science, Ubon Ratchathani University, Ubon Ratchathani, 34190, Thailand*
[b] *College of Medicine and Public Health, Ubon Ratchathani University, Ubon Ratchathani, 34190, Thailand*
[c] *Department of Chemistry, Faculty of Science, Ubon Ratchathani University, Ubon Ratchathani, 34190, Thailand*
[d] *Division of Chemistry, Faculty of Science, Nakhon Phanom University, Nakhon Phanom, 48000, Thailand*
[e] *National Nanotechnology Center, NSTDA, 111 Thailand Science Park, Klong Luang, Pathum Thani, 12120, Thailand*

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ABSTRACT

Background: Iron overload and inflammation are severe conditions that can lead to various chronic diseases. However, the current iron chelator drugs have their limitations. The phytochemical compounds from herbals, such as brazilin, the major active compound in *Caesalpinia sappan* Linn., have significant therapeutic potential in various chronic diseases. Our study was designed to examine the effect of brazilin on iron chelating properties, antioxidant activity in hepatocytes, and anti-inflammatory potential in macrophages.

Methods: This study focused on the isolation, purification, and evaluation of brazilin, the principal bioactive constituent found in *C. sappan* wood. Brazilin was extracted *via* methanol maceration followed by column chromatography purification. The purified compound was characterized using high-performance liquid chromatography (HPLC), nuclear magnetic resonance (NMR) spectroscopy, and mass spectrometry (MS). The antioxidant potential of brazilin was assessed by in *vitro* assays, including 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azinobis-(3-ethylbenzthiazolin-6-sulfonic acid (ABTS), and ferric-reducing antioxidant power (FRAP). Furthermore, its cellular antioxidant activity was evaluated using hydrogen peroxide-induced oxidative stress in the hepatocellular carcinoma cell line (Huh-7). The iron-chelating capacity of brazilin was determined spectrophotometrically, and Job's plot method was used to elucidated the stoichiometry of the iron-brazilin complex formation. The anti-inflammatory properties of brazilin were investigated in lipopolysaccharide (LPS)-stimulated macrophages (RAW 264.7). Nitric oxide (NO) inhibition was quantified using the Griess reagent, while the expression levels of pro-

Abbreviations: C. sappan, Caesalpinia sappan Linn.; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; MS, mass spectrometry; DPPH, 2,2-diphenyl-1-picrylhydrazyl; ABTS, 2,2′-azinobis-(3-ethylbenzthiazolin-6-sulfonic acid; FRAP, ferric-reducing antioxidant power; Huh-7, hepatocellular carcinoma cells; LPS, lipopolysaccharide; RAW 264.7 cell, macrophage cells; NO, Nitric oxide; IL-6, interleukin 6; TNF-α, tumor necrosis factor-alpha.

* Corresponding author.
E-mail address: kanjana.pa@ubu.ac.th (K. Pangjit).

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inflammatory cytokines, interleukin-6 (*IL-6*) and tumor necrosis factor-alpha (*TNF-α*), were evaluated by RT-qPCR.
Results: The results demonstrated that brazilin exhibited potent antioxidant activity in *vitro* and hepatocytes in a concentration-dependent manner. It also showed anti-inflammatory activity, in which NO production was significantly reduced and *IL-6* and *TNF-α* expression in LPS-induced macrophages were repressed. Furthermore, it can bind ferric and ferrous ions. Brazilin acts as a bidentate iron chelator that forms a complex with iron in a 2:1 ratio, and two water molecules are used as additional chelators in this complex.
Conclusions: Our findings have significant implications. Brazilin can potentially alleviate the harmful effects of iron-induced oxidative stress and inflammatory disorders.

1. Introduction

Iron is an essential trace element for cellular metabolism and a heme component, such as hemoglobin, myoglobin, cytochrome, and iron-sulfur cluster [1]. Iron-containing proteins play a significant role in oxygen transport, electron transport system, detoxification, DNA replication, and repair [2]. Iron is a transition metal in the human body, which binds to transferrin, a major iron-binding protein, to protect tissues from oxidative damage in blood circulation. Ineffective erythropoiesis increases intestinal iron absorption, and multiple blood transfusions in thalassemia develop into iron overload [3]. Excessive iron, non-transferrin bound iron (NTBI), can be taken up by cells and accumulate in various tissues [4]. It can generate reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), *via* the Haber-Weiss and Fenton reactions and convert H_2O_2 into hydroxyl radicals ($OH^{\bullet}$) [5]. ROS can damage various cellular macromolecules, such as nucleic acids, proteins, and membrane lipids. These radicals harm many cellular and other tissues, such as the heart, liver, pancreas, erythrocytes, and endocrine glands, resulting in organ dysfunction and causing fibrosis, cirrhosis, cardiomyopathy, and diabetes mellitus [6,7]. Effective iron chelators are necessary to remove toxic iron and prevent oxidative damage to vital organs, particularly the heart and liver. Furthermore, iron loading is associated with chronic inflammation, such as diabetes [8] and chronic liver disease [9].

Inflammation is a defensive mechanism in the immune system to protect cells or tissue from harmful stimuli, such as pathogens, or allergic and chemical irritations [10]. In the normal process of inflammation, macrophages produce pro-inflammatory cytokines, including interleukin 6 (IL-6) and tumor necrosis factor-α (TNF-α), as well as nitric oxide (NO) and prostaglandins (PGs), at the inflammatory site for healing process [11]. Nitric oxide is generated by the L-arginine precursor and is catalyzed by nitric oxide synthase (NOS) in macrophages [12]. However, dysregulated inflammation, such as excessive NO production, mediates acute and chronic inflammation [13]. The overproduction of inflammatory mediators causes many inflammatory diseases. Anti-inflammatory agents can protect against these diseases.

Natural products (NPs) are the sources of potential therapeutic drugs due to their diverse pharmacological properties, including anti-inflammatory and antioxidant effects. *Caesalpinia sappan* L. (*C. sappan*) is a traditional medicinal plant in Southeast Asia. Its phytochemicals have been used as herbal medicines with many bioactivities, such as anticancer, antimicrobial [14,15], anti-inflammatory, antioxidant [15], hypoglycemic, and neuroprotective effects [16,17]. Bioactive compounds isolated from *C. sappan* include diterpene, homoisoflavone, and polyphenolics [18]. Brazilin is a major bioactive compound in a group of homoisoflavones in *C. Sappan.* NPs have bifunctional properties against oxidative stress and inflammation in chronic diseases.

Numerous iron chelators treat thalassemia in patients with iron overload [19–21]. In addition to these commercial medicines, some polyphenols and flavonoids exhibit iron-chelating properties [22–24]. Several chelators have been developed and evaluated for their efficiency and toxicity in *vitro* and in *vivo*. Ethanolic extract of *C. sappan* wood exhibits iron chelation in iron overload rats [25], but the effective bioactive compound of these *C. sappan* remains unknown. Interestingly, the major active compound in *C. sappan* is brazilin, which has a catechol group on the structure, leading to a high efficiency of iron chelating activity [23]. However, the properties of brazilin's iron-binding activity have not been elucidated. Based on the mentioned evidence, this study aimed to examine the iron-chelating, antioxidant, and anti-inflammatory activities of the purified brazilin from *C. Sappan.*

2. Materials and methods

2.1. Plant material

The heartwoods of *Caesalpinia sappan* (*C. sappan*) were collected from Renu Nakhon district, Nakhon Phanom Province, Thailand, in July 2021. The *C. sappan* collection complied with the guidelines and regulations of Thailand's legislation. The botanical identification was confirmed by Assistant Professor Dr. Sawai Mathapha, Department of Biological, Faculty of Science, Udon Thani Rajabhat University. A voucher specimen (P. Kamsri0001; KKU 25766) has been deposited in the herbarium of the Khon Kaen University Plant Museum, Department of Biology, Faculty of Science, Khon Kaen University. Taxonomic information of sappan is listed in the Integrated Taxonomic Information System (ITIS) report (taxonomic serial number: 506349).

2.2. Extraction and isolation of brazilin

The air-dried heartwoods of *C. sappan* (10 kg) were pulverized to powder using a grinder, and the powder was passed through sieve

No. 50. The sappanwood powder was macerated with methanol (MeOH) (30 L) for 5 days at room temperature. This process was repeated three times with the same volume of solvent and the resulting suspension was filtered (Whatman No. 2). The solvent was removed under reduced pressure by using rotary evaporator at 40 °C to yield dark reddish brown solid. The percentage yield of MeOH extract was found to be 6.35 % w/w. An aliquot (100 g) of concentrated methanol extract was fractionated by hexane and ethyl acetate and the resulting fractions were evaporated to dryness to give hexane extract and ethyl acetate extract at 1.5 % and 5.6 % (w/w) yield, respectively. The obtained extracts were finally powdered and stored in a refrigerator for further use. Based on iron chelating screening, and brazilin content determination of *C. sappan* crude extract, both methanolic and ethyl acetate extracts showed iron chelating activity. However, the methanolic extract has higher amount of brazilin than ethyl acetate extract (section 3.1). Thus, the methanolic extract was chosen for further isolation and purification. The methanolic extract (10 g) was dissolved in methanol and mixed with silica gel (5 g, mesh size 60–120) to make a dry sample for convenient application in quick column chromatography (QCC). The dry sample was then loaded on a clean and dry glass column (4*40 cm) packed with silica gel (60–120 mesh size, 1 g extract per 50 g silica gel) and eluted with dichloromethane, dichloromethane: ethyl acetate (1 : 1), ethyl acetate, ethyl acetate: methanol (1 : 1), and methanol, successively. This separation yielded five major fractions (A1-A5) based on thin-layer chromatography (TLC) analysis. The fraction A2 and A3 were combined (A2-3, 5.27 g) and the resulting mixture was further separated by silica gel column chromatography (CC) (3*40 cm) and eluted with a gradient solvent system of dichloromethane-ethyl acetate (10:0 to 0:10). Each 10 mL fraction was collected and monitored by TLC under UV light at 254 nm. The TLC was also stained with p-anisaldehyde reagent to clearly identify the composition. Similar fractions were combined to produce ten fractions (B1-B10). Fractions B7 (2.58 g) were re-chromatographed on silica gel column chromatography (3*25 cm), using dichloromethane-ethyl acetate (7 : 3, 6 : 4, 1 : 1, 4 : 6) and ethyl acetate as eluents to afford brazilin (65 mg), which was identified by NMR analysis. The extraction and purification procedures of brazilin from *C. sappan* were shown in Scheme 1.

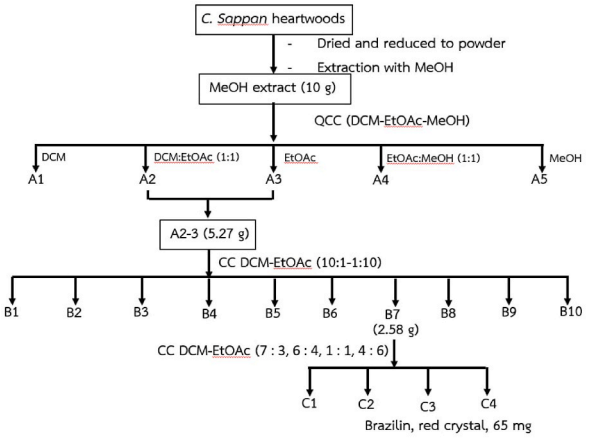
2.3. Nuclear magnetic resonance (NMR) spectroscopic and mass spectrometric (MS) analysis

The structural determination was analyzed by NMR spectra, which were recorded in CD_3OD(Cambridge Isotope Laboratories, Massachusetts, U.S.A) on a Varian Mercury Plus 400 spectrometer (Varian Inc., USA) using the residue of this solvent as the internal standards. Data were reported as follows: chemical shifts, multiplicity, coupling constant. Multiplicities in the ^{1}H NMR spectra were described as: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad; coupling constants (*J*) were reported in Hz. High-resolution mass spectra (HR-MS) were recorded using Bruker micrOTOF mass spectrometer (ESI-TOF) and reported with ion mass/charge (*m/z*) ratios as values in atomic mass units.

2.4. Purity and brazilin determination via high-performance liquid chromatography (HPLC)

HPLC (DIONEX, Ultimate 3000) was performed to analyze the purity of purified brazilin from *C. sappan* [26]. The standard brazilin (Chemface©), ethyl acetate crude extract, methanolic crude extract and purified brazilin were dissolved in methanol and filtrated through a 0.45 μm membrane before injection. The samples were analyzed with reverse-phase HPLC using a C-18 column (4.6 mm × 250 mm, 5 μm particle diameters, Acclaim™ 120). The mobile phase consisted of two solvents: 0.1 % trifluoroacetic acid in water (solvent A) and methanol (solvent B). Gradient elution was performed in the following: 10 % solvent B (methanol); 0–15 min, 60 % solvent B; 15–25 min, 90 % solvent B; 25–55 min, and transferred to initial conditions (10 % solvent B). Ten microliters of each standard and sample were injected into the column at a 1.0 mL/min flow rate and detected at 280 nm. The amounts of brazilin in crude extracts were calculated by using the area of the brazilin peak. The purity of purified brazilin was calculated using the following equation:

% purity = (peak area of purified brazilin × 100)/ total peak area.



Scheme 1. The extraction and purification procedures of brazilin from *C. sappan*.

2.5. Iron-binding assay

Various concentrations of ferric nitrilotriacetate (Fe^{3+}-NTA) or ferrous ammonium sulfate hexahydrate ($Fe(NH_4)_2(SO_4)_2.6H_2O$) at pH 7.0 were incubated with *C. sappan* crude extract (500 μg/mL at final concentration) or brazilin (100 μM at final concentration) for 60 min. The absorbance of the complexes was continuously measured in the wavelength range of 200–900 nm using a UV–VIS spectrophotometer [27], and 500 μg/mL of the extract or 100 μM of the brazilin solution was used as a blank. A stock solution of ferrous ammonium sulfate was freshly prepared by dissolving it in a 50 mM MOPS solution at pH 7.0. The working Fe^{3+}-NTA solution was freshly prepared by mixing a stock standard iron solution with nitrilotriacetate (NTA) solution (molar ratio of Fe^{3+}: NTA = 1:5).

2.6. The molar ratio of iron to brazilin using Job's method

Job's method was used to determine the stoichiometry of the iron–brazilin complexes. In this method, the total molar concentrations of iron and purified brazilin were kept constant, but the molar fractions varied. Different ratios of ferric ions (Fe^{3+}-NTA) and brazilin were prepared while maintaining the total concentration of the metal and ligand at 0.2 mM. A 50 mM MOPS buffer containing 50 % methanol (v/v) was used to maintain a pH of 7.0. The absorbance of the complexes was measured at 590 nm using a spectrophotometer [28]. The absorption of the iron chelator complex at 590 nm was plotted against the molar ratios of the two components. The maximum of the corresponding Job's plot indicates the stoichiometry (1:1 complexation is indicated by a maximal value at 0.5, 1:2 complexation is indicated by a maximal value at 0.66, and 1:3 complexation is indicated by a maximal value at 0.33).

2.7. Molecular docking

Molecular docking calculations were performed using the Autodock 4.2 program [29] to model the structure of the ferric–brazilin complex. The coordinates of ferric ions and brazilin were constructed using Gaussview 5.0 program [30] and then optimized using the B3LYP/6-31G* basis set implemented in Gaussian 09 [31]. Thirty-four grid points in the x, y, and z dimensions with a grid point spacing of 0.375 Å were used to define the 3D grid box centered on the ferric ion coordinate. Except for the number of GA runs, which was set to 500, default search algorithm parameters were employed. A Gasteiger charge was used for the ferric ions and brazilin. During the molecular docking calculations, the ferric ions remained rigid, whereas the brazilin structures were flexible.

2.8. Antioxidant assay

2.8.1. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

The free radical scavenging activity of brazilin was determined using a DPPH radical assay with slight modifications [32]. Briefly, 20 μL of brazilin (1 mg/mL of stock solution in methanol) was incubated with 180 μL of 80 mM methanolic DPPH radical solution for 30 min in the dark. The absorbance of the DPPH solution was measured at 517 nm using a microplate reader spectrophotometer (Biochrom - EZ Read 2000). All experiments were performed in triplicates. The following equation was used to calculate the percentage of DPPH radical scavenging activity:

$$\%\ \text{DPPH scavenging} = \left(OD_{control} - OD_{sample\ or\ trolox}\right) / OD_{control} \times 100$$

2.8.2. 2,2′-azinobis-(3-ethylbenzthiazolin-6-sulfonic acid (ABTS) assay

The antioxidant activity against the $ABTS^{\bullet+}$ radical was measured according to Bednarska *et al.* with slight modification [33]. ABTS (7 mM) and potassium persulfate (2.45 mM) were prepared in deionized water. Two solutions were mixed at a 1:1 ratio and the resulting solution was incubated in the dark for 12 h and diluted with methanol to obtain an absorbance of 0.7 ± 0.02 at 734 nm. Two microliters of brazilin solution were mixed with 198 μL of $ABTS^{\bullet+}$ radical and the resulting mixture was incubated in the dark at room temperature for 6 min. Absorbance was measured at 734 nm using a microplate reader spectrophotometer. Trolox was used as the positive control. All experiments were performed in triplicates. ABTS radical scavenging activity was calculated as follows:

$$\%\ \text{ABTS scavenging} = \left(OD_{control} - OD_{sample\ or\ trolox}\right) / OD_{control} \times 100$$

The EC_{50} value (the effective concentration of brazilin that reduces initial DPPH radical or $ABTS^{\bullet+}$ radical by 50 %) was obtained by interpolation from linear regression analysis and used to express the antioxidant activity.

2.8.3. Ferric reducing antioxidant power (FRAP) assay

FRAP assay was performed according to the method described by Benzie and Strain with some modifications [34]. Working FRAP reagent was prepared at a ratio of 300 mM acetate buffer: 10 mM TPTZ in 40 mM HCl: 20 mM $FeCl_3$ (10:1:1). Briefly, 10 μL of brazilin (1 mg/mL of stock solution in methanol) was incubated with 190 μL of working FRAP reagent for 30 min. The absorbance was measured at 593 nm using a microplate spectrophotometer. Trolox was used as the standard. The results were expressed as milligrams of Trolox equivalent to grams of brazilin.

2.9. Cell culture

Hepatocellular cells (Huh7) and murine macrophage cell lines (RAW 264.7), primary dermal fibroblast cells (HDFn), and kidney

epithelial cells (NRK-52E) were purchased from the American Type Culture Collection (ATCC; TIB-71, VA, USA). Cells were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10 % (v/v) inactivated fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin (Pen-strep), and maintained at 37 ^{O}C in a 5 % CO_2 incubator.

2.10. Cell viability assay

Cell viability was determined using the MTT assay. Briefly, Huh7 cells ($5x10^3$ cells/well) or RAW 264.7 cells ($1x10^4$ cells/well), HDFn cells ($1x10^4$ cells/well) or NRK 52E cells ($1x10^4$ cells/well) were cultured in 96-well plates and treated with various brazilin concentrations for 24 h. After changing the medium, MTT solution (5 mg/mL) was added to the cells and further incubated for 4 h at 37 °C. The medium was then removed and DMSO was added to dissolve the formazan crystals. Finally, the absorbance was measured at 540 nm. Untreated cells were used as controls to calculate the percentage of viable cells after treatment.

2.11. Assessment of intracellular ROS level in hepatocytes

The intracellular ROS level in Huh7 cells was determined using 2′,7′- dichlorofluorescein diacetate (DCFH-DA) assay. Huh7 cells were incubated with and without brazilin (4 and 8 μM) for 24 h, the medium was removed, and the cells were washed with phosphate-buffered saline at pH 7.4. The treated cells were incubated with 10 μM DCFH-DA for 30 min at 37 ^{O}C in a 5 % CO_2 incubator. After washing, the cells were incubated with 125 μM hydrogen peroxide (H_2O_2) for 20 min at 37 ^{O}C in a 5 % CO_2 incubator. ROS production was measured using a fluorescent microplate reader at excitation/emission wavelengths of 485/530 nm [35]. The fluorescent intensity was directly proportional to the intracellular ROS levels.

2.12. Assessment of nitric oxide production in macrophage cells

The murine macrophage RAW 264.7 cells were seeded in six-well plates at a density of 7×10^5 cells/well and incubated at 37 °C for 24 h. First, brazilin (4, 8, and 16 μM) or N(gamma)-nitro-L-arginine methyl ester (L-NAME, 50 μg/mL) as a positive control was added to the cell culture medium and mixed gently. LPS (2 μg/mL) was added to the cells and incubated for 24 h. The supernatant media were collected and incubated with Griess reagent comprising 0.1 % (w/v) naphthyl-ethylene-diamide dihydrochloride, 1 % (w/v) sulfanilamide, and 5 % (v/v) concentrated phosphoric acid, and absorbance was measured at 540 nm using a spectrophotometer. The amount of nitrite, a nitric oxide metabolite, was calculated from the standard curve of sodium nitrite (0–40 μM).

2.13. Assessment of pro-inflammatory gene expression in macrophage cells using RT-qPCR

RAW 264.7 cells were seeded in six-well plates at a density of 7×10^5 cells/well and incubated at 37 °C for 24 h. The cells were then treated as described previously. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Total RNA was quantified before reverse transcription into cDNA, using a high-capacity cDNA Reverse Transcription Kit (Applied Biosystems). The template cDNA used for RT-qPCR amplification was LightCycler® 480 SYBR Green I Master (Roche, USA). The pro-inflammatory mediator primers were obtained from Macrogen (Macrogen Company, Seoul, Korea), and the sequences are shown in Table 1. The RT-qPCR reaction conditions were 45 cycles at 95 °C for 10 min, 60 °C for 15 min, and 72 °C for 10 min. The target mRNA was quantitatively analyzed using the equation: relative quantitation (RQ) $= 2^{-\Delta\Delta Ct}$. Target mRNA levels were calculated relative to those of the untreated control group and normalized to those of GAPDH.

2.14. Statistical analyses

Each experiment was performed in triplicates. The results are expressed as mean ± standard error of the mean (SEM). Statistics were performed using a one-way analysis of variance (Tukey's test) comparing all study groups (95 % confidence intervals). Statistical significance was set at $P < 0.05$.

Table 1
Primer sequences of pro-inflammatory mediator and house-keeping gene in RT-qPCR.

Gene	Sequences	PCR product (bp)
TNF-α	Forward: 5′ AGAAAGCATGATCCGCGACG 3′ Reverse: 5′TTTGCTACGACGTGGGCTAC 3′	285
IL-6	Forward: 5′GACAAAGCCAGAGTCCTTCAGAG 3′ Reverse: 5′GGTCTTGGTCCTTAGCCACTC 3′	159
GAPDH	Forward: 5′TCCCTGAGCTGAACGGGAA 3′ Reverse: 5′CGATGCCTGCTTCACTACCT 3′	138

3. Results

3.1. Quantification and characterization of brazilin

The amount of brazilin in *C. sappan* crude extracts was analyzed using HPLC. The chromatogram profile of the crude extract showed the presence of brazilin at a retention time of 12.8 min (Fig. 1). Brazilin contents in ethyl acetate and methanolic extracts were 145.59 ± 3.72 and 155.98 ± 2.34 mg/g dry weight, respectively. The identification of brazilin was performed by nuclear magnetic resonance (NMR) spectroscopy for structural determination, high-resolution electrospray ionization mass spectrometry (HR-ESIMS) for molecular mass calculation, and high-performance liquid chromatography (HPLC) for purity analysis of purified brazilin. Brazilin was red crystals, ^{1}H NMR (400 MHz,CD_3OD): δ 2.81 (1H, d, J = 15.7 Hz, H-7b), 3.01 (1H, d, J = 15.7 Hz, H-7a), 3.71 (1H, d, J = 11.2 Hz, H-6b), 3.92 (1H, dd, J = 1.2 Hz, J = 11.2 Hz, H-6a), 3.96 (1H, s, H-12), 6.29 (1H, d, J = 2.4 Hz, H-4), 6.49 (1H, dd, J = 8.2 Hz, J = 2.4 Hz, H-2), 6.65 (1H, s, H-11), 6.76 (1H, s, H-8), 7.20 (1H, d, 8.0, H-1), (Fig. 2A), ^{13}C NMR (100 MHz, CD_3OD): δ 42.8 (C-4a), 51.1 (C-3), 70.7 (C-9), 77.6 (C-10), 103.9 (C-11a), 109.5 (C-1), 112.2 (C-7a), 112.6 (C-1a), 115.5 (C-8), 131.4 (C-11), 131.9 (C-2), 137.3 (C-4), 145.1 (C-6a), 144.7 (C-6), 155.4 (C-12), 157.5 (C-7) (Fig. 2B), HR-ESIMS: *m/z* 309.0737 $[M+Na]^+$ (Fig. 2C), and the chemical structure of brazilin was shown in Fig. 2D. The HPLC chromatogram showed the purity of brazilin of 89.79 ± 0.22 % (Fig. S1). The ^{1}H and ^{13}C NMR spectra in this study were found to be consistent with brazilin, as previously reported [36].

3.2. Iron-binding property

The metal ion-binding activity of *C. sappan* crude extract and brazilin was determined *via* UV–VIS spectroscopy. The ethyl acetate and methanolic extracts of *C. sappan* exhibited iron binding properties and showed the maximum absorbance at 400 and 590 nm (Fig. 3A and B). Brazilin has iron-binding properties with both ferric and ferrous ions. The complexes resulted in a red-purple solution. The predominant peaks of the brazilin–ferric complexes were at 240, 305, and 590 nm (Fig. 3C). The absorbance of the brazilin–ferric complexes increased in a dose-dependent manner with ferric ion concentrations being in the range of 12.5–200 μM. Similar to the ferrous ions, the maximum absorption bands of the brazilin–ferrous complex solution were 240, 305, and 590 nm. The absorbance of the complexes increased dose-dependently with ferrous ion concentration being in the range of 12.5–100 μM (Fig. S2).

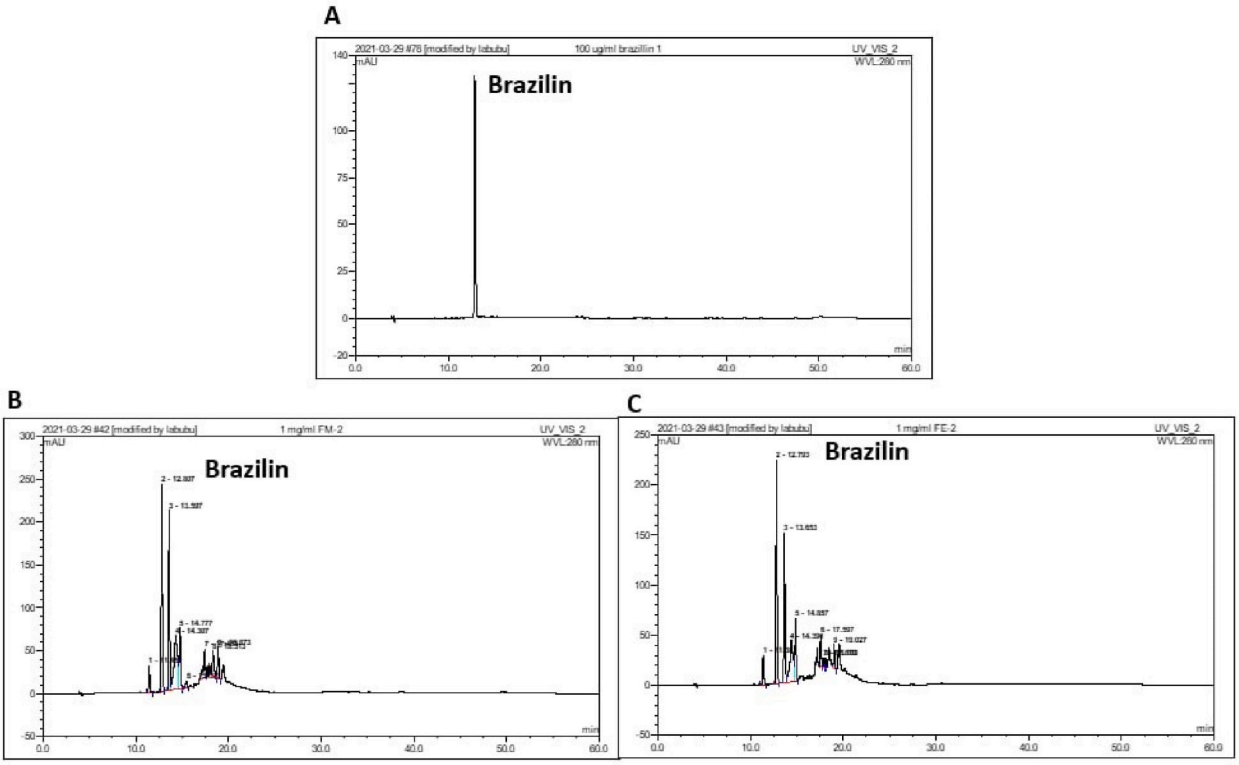


Fig. 1. HPLC chromatogram of brazilin standard (retention time = 12.8 min) (A), methanolic crude extract (B), and ethyl acetate crude extract (C) of *C. sappan* at 280 nm.

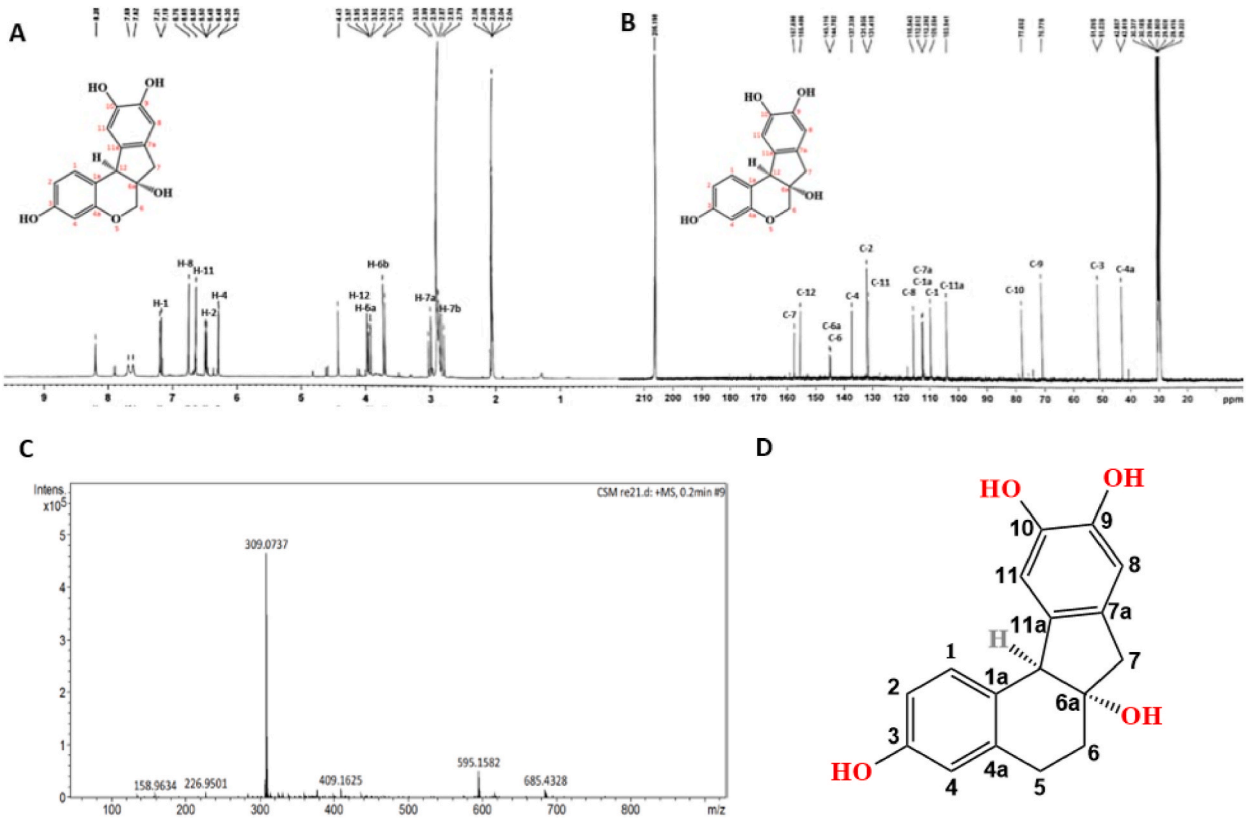


Fig. 2. Brazilin was isolated from *C. sappan* ^{1}H NMR (A), ^{13}C NMR spectrum (B), brazilin MS data (C), and chemical structure of brazilin (D).

3.3. Molar ratio of iron-binding

The maximum of the corresponding Job's plots indicated that the stoichiometry presents a 2:1 complexation (brazilin: ferric ion), characterized by the maximal value occurring at 0.66 (Fig. 4).

3.4. Brazilin–ferric ion complexation

Molecular docking calculations showed that brazilin was a bidentate chelator. Furthermore, 2:1 complexation (brazilin: ferric ion) was modeled and geometrically optimized by DFT (B3LYP/6–31 G*) using Gaussian 9.0 (Fig. 5). As the brazilin–ferric ion complex is assumed to be octahedral, two water molecules were used as additional chelators in this complex, providing a comprehensive and accurate representation of the interaction.

3.5. Antioxidant property

DPPH, a stable free radical, was evaluated for the scavenging activity of brazilin. The half-maximal effective concentration values (EC_{50}) of the antioxidant activity of brazilin and trolox were 83.62 ± 3.98 and 8.50 ± 0.74 μM, respectively. Similarly, brazilin and trolox could inhibit the stable $ABTS^{\bullet+}$ radicals with EC_{50} values of 28.15 ± 1.99 and 16.18 ± 0.42 μM, respectively. The ferric-reducing antioxidant power assay measured the reducing potential of brazilin reacting with ferric ion to ferrous ion. The reducing activity of brazilin and trolox was 10.80 ± 0.34 and 55.80 ± 1.00 μM Fe^{2+}/μg brazilin, respectively.

3.6. Cytotoxicity of brazilin

Cell viability assay was performed to determine the cytotoxicity of brazilin. Brazilin was significantly cytotoxic at 32 μM and 16 μM in RAW 264.7 and Huh 7 cells, respectively (Fig. 6A and B). The results indicated that brazilin has more cytotoxicity in Huh 7 cells than that in RAW 264.7 cells. The concentrations of brazilin below 16 μM and 8 μM were non-toxic to RAW 264.7 and Huh 7 cells, respectively, so this concentration could be used in the following experiments. Additionally, brazilin concentrations of 4–16 μM showed no toxicity to normal cells, including primary dermal fibroblast (HDFn cells) (Fig. 6C) and kidney epithelial cells (NRK-52E cell) (Fig. 6D).

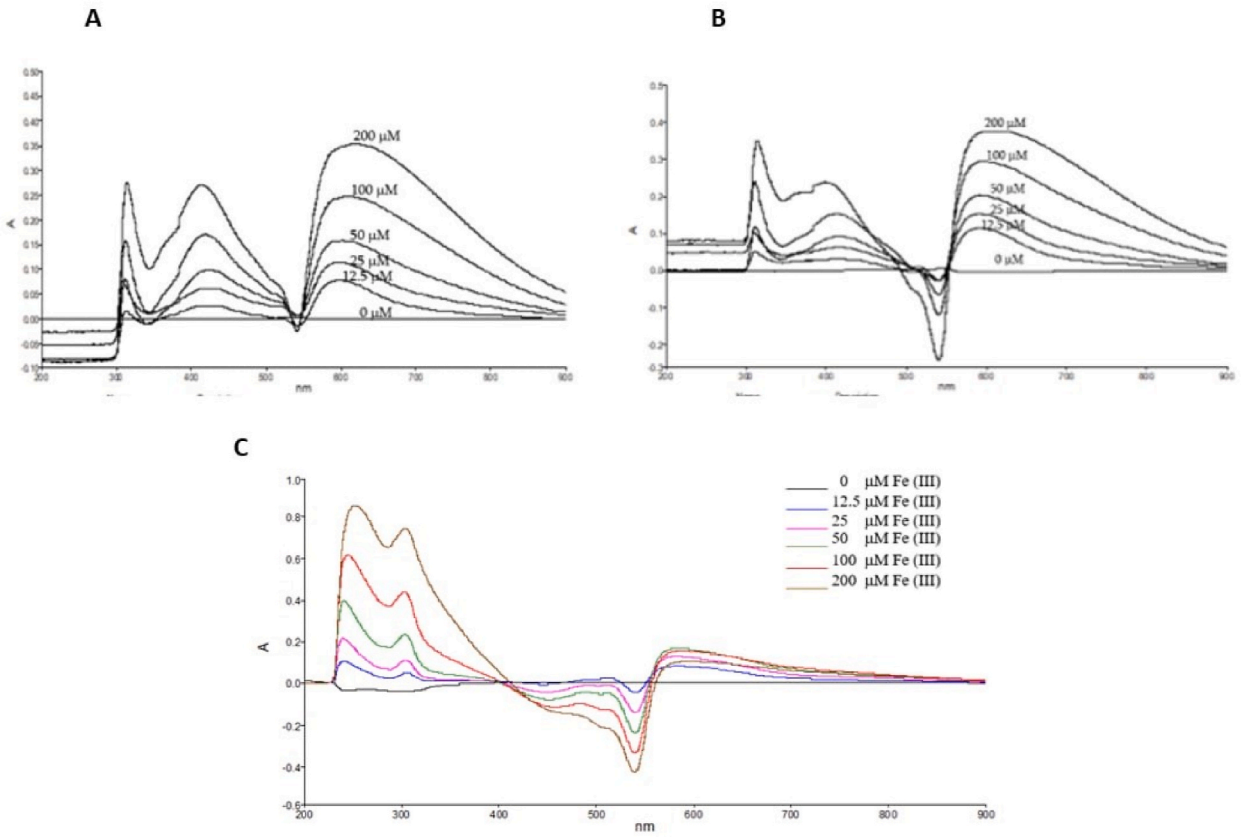


Fig. 3. The absorption spectrum of ethyl acetate extract-ferric ion complex (A), methanolic extract-ferric ion complex (B), and brazilin–ferric ion complex (C) at 200–900 nm. Ferric nitrilotriacetate (0–200 μM) were incubated with 500 μg/mL extracts or 100 μM brazilin for 60 min. The extracts or brazilin–iron complexes were measured at a wavelength range of 200–900 nm using a UV–VIS spectrophotometer.

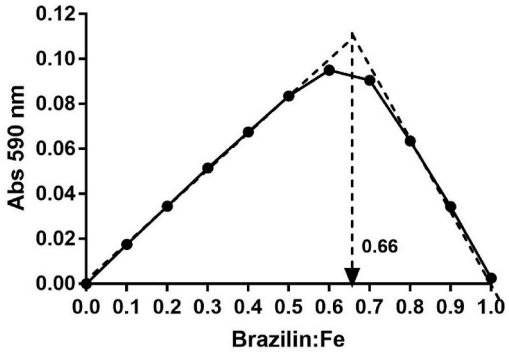


Fig. 4. The molar ratio of brazilin–ferric ion complexes. The molar ratios of ferric ion (Fe^{3+}-NTA) and brazilin were prepared while keeping the total concentration of metal ion plus ligand at 0.2 mM. The absorption value of the complex was measured using a spectrophotometer at 590 nm.

3.7. Antioxidant in hepatocytes

The DCFH-DA assay was performed to determine whether brazilin can scavenge ROS in Huh 7 cells. Hydrogen peroxide treatment induced ROS production compared to untreated cells. Brazilin (4 and 8 μM) treatments significantly decreased, in a dose-dependent manner, the H_2O_2-stimulated ROS production. The positive antioxidant control, trolox (10 μg/mL), significantly limited the ROS production on H_2O_2-treated Huh 7 cells (Fig. 7).

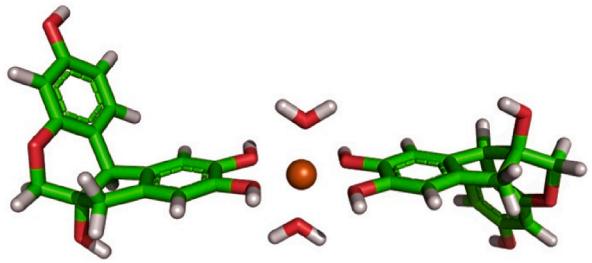


Fig. 5. The binding of brazilin to ferric ions was obtained from molecular docking calculations. Optimized geometry of octahedral ferric–brazilin complex. Carbon, oxygen, hydrogen, and ferric ions are indicated by green, red, white, and brown colors, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

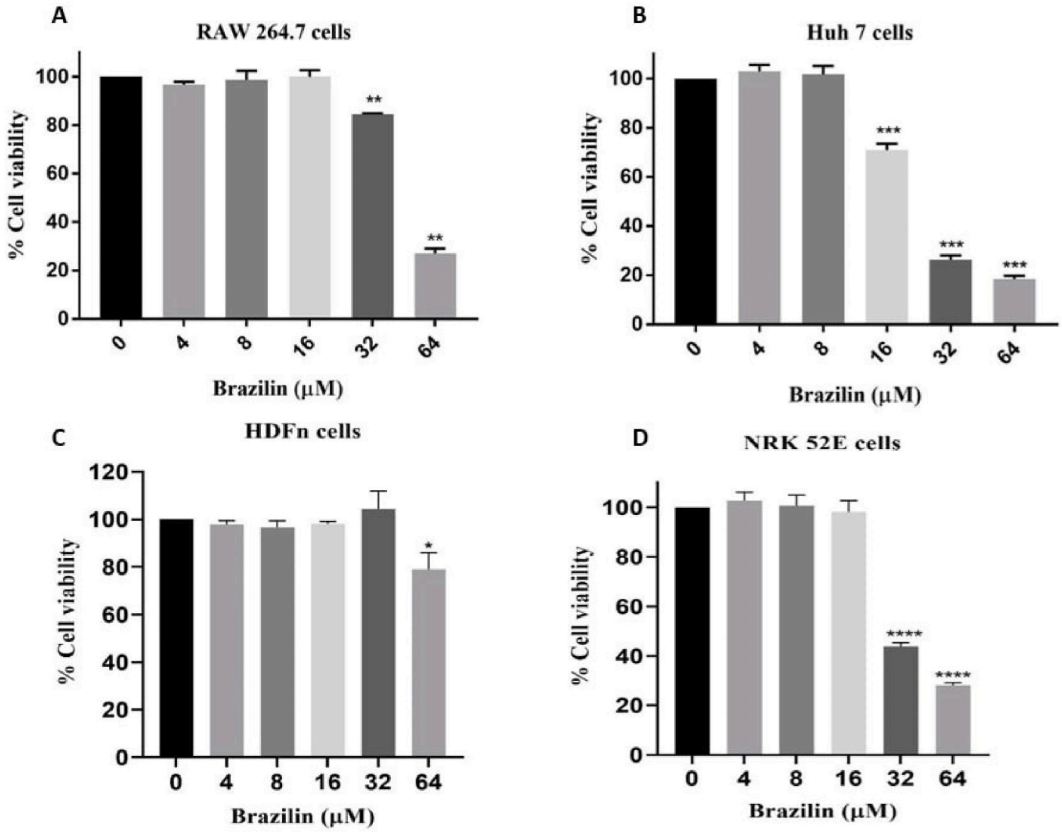


Fig. 6. Cell viability of RAW 264.7 cells (A) and Huh 7 cells (B), HDFn cells (C), and NRK 52E cells (D) treated with brazilin (0–64 μM) for 24 h. Data obtained from three independent experiments are expressed as mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$ when compared with non-treated cells.

3.8. Anti-nitric oxide production in macrophage cells

The RAW 264.7 cells were exposed to various concentrations of brazilin and induced with LPS (2 μg/mL). The nitric oxide (NO) production was measured as nitrite concentration. The amount of nitrite significantly increased when exposed to LPS, and treatments of positive control (L-NAME, 50 μg/mL) and brazilin (4, 8 and 16 μM) significantly decreased the LPS-induced NO production in RAW264.7 cells (Fig. 8). Treatment of brazilin in LPS-stimulated RAW 264.7 cells could significantly suppress the secretion of NO.

3.9. Anti-inflammatory activity in LPS-stimulated macrophage cells

The inhibitory effect of brazilin on inflammatory cytokines mRNA expression, including *IL-6* and *TNF-α* in LPS-treated RAW 264.7 cells was determined using RT-qPCR. LPS treatment significantly upregulated the expression of IL-6 and TNF-α in RAW 264.7 cells,

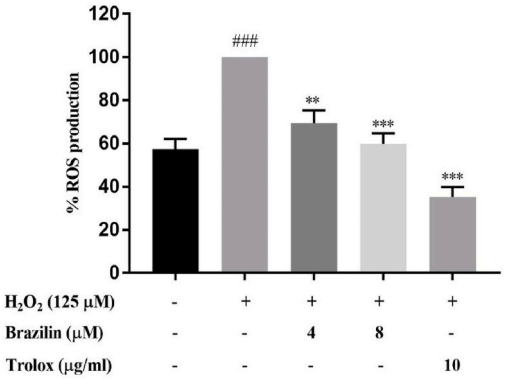


Fig. 7. Reactive oxygen species (ROS) in Huh-7 cells treated with 10 μg/mL trolox or brazilin (4 and 8 μM) for 24 h. Trolox was used as the positive reference. Data obtained from three independent experiments are expressed as mean ± SEM. $^{###}p < 0.001$ when compared with H_2O_2 untreated cells, $^{**}p < 0.01$ and $^{***}p < 0.001$ when compared with non-treated cells.

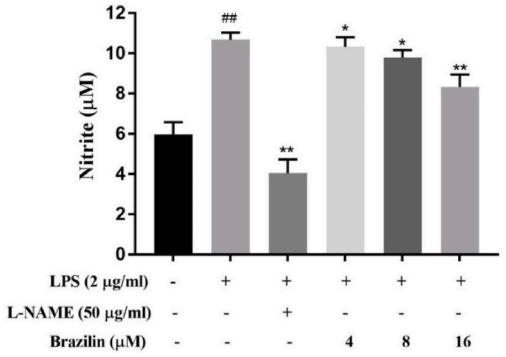


Fig. 8. Nitrite levels in RAW 264.7 cells treated with LPS (2 μg/mL) and L-NAME (50 μg/mL) or brazilin (4, 8, and 16 μM) for 24 h. L-NAME was used as the positive reference. Data obtained from three independent experiments are expressed as mean ± SEM. $^{##}p < 0.01$ when compared with LPS untreated cells, $^{*}p < 0.05$ and $^{**}p < 0.01$ when compared with the non-treated cells.

whereas positive control (L-NAME, 50 μg/mL) significantly downregulated IL-6 and TNF-α mRNA expression in LPS-treated RAW 264.7 cells (Fig. 9A and B). In addition, brazilin (4, 8 and 16 μM) could significantly decrease IL-6 mRNA levels dose-dependently (Fig. 9A). The level of TNF-α mRNA in LPS-treated cells was not significantly changed by brazilin treatment (4 and 8 μM). Treatment with 16 μM brazilin significantly decreased TNF-α mRNA levels in LPS-treated cells (Fig. 9B).

4. Discussion

Phytochemicals, such as polyphenolics and flavonoids from herbs, are gaining increasing attention owing to their therapeutic potential as antioxidants, iron-binding agents, and anti-inflammatory agents. Brazilin is the main active compound isolated from *C. sappan* [37]. Brazilin is a homoisoflavonoid belonging to a subclass of flavonoids originating from the chalcone precursor ring [14]. Recently, there have been several reports for brazilin extraction and purification from *C. sappan* heartwood, such as one-step purification of the ethanolic extract with a macroporous resin column [38] and repeated preparative HPLC separation of the ethanolic extract. Methanol, ethanol, and water extracts showed antioxidant potential [39]. This study aimed to extract brazilin from *C. sappan* heartwood using methanol, followed by sequential separation and purification *via* column chromatography. Brazilin was then identified using HR-ESI-MS for molecular mass calculations and NMR for structural determination. It has a molecular formula of $C_{16}H_{14}O_5$ and a molecular ion at *m/z* 309.07. The purity of purified brazilin was 89.79 ± 0.22 %, as confirmed using analytical HPLC. Brazilin exhibits iron-chelating properties towards both ferrous and ferric ions. Moreover, ferrous ions can initiate free radical generation, and the chelation of ferrous ions can prevent the metal catalysis of ROS generation. Brazilin's structure contains a catechol functional group in ring B capable of chelating the six available orbitals of ferric ions (Fe^{3+}) in a 2:1 ratio according to Job's method under neutral conditions. According to molecular docking calculations, brazilin was a bidentate chelator, and two water molecules were used as chelators in the brazilin–ferric complex. The most appropriate iron-binding site for brazilin is the catecholic ring B [23,40]. In addition, 9,10 dihydroxy is the most effective iron-binding site for brazilin. The polyhydroxy and carbonyl groups of flavones are potential sites for iron chelation. Other flavonoids, such as rutin and quercetin, can chelate ferric ions (Fe^{3+}) in the ratio (flavonoid: ferric ion) 1:1 and 2:1, respectively, under neutral conditions [41]. The presence of three or five hydroxy groups on rutin and quercetin structures is

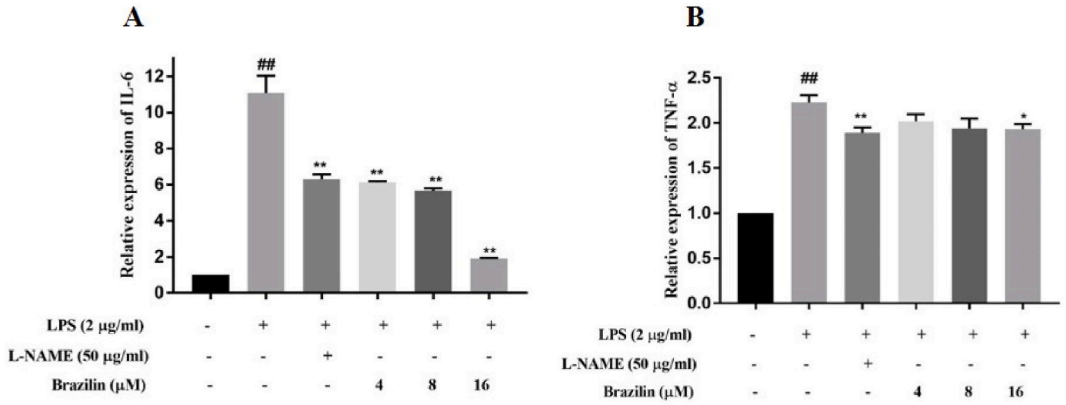


Fig. 9. The relative mRNA expression of IL-6 (A) and TNF-α (B) in RAW 264.7 cells treated with LPS (2 μg/mL) and L-NAME (50 μg/mL) or brazilin (4, 8, and 16 μM) for 24 h. L-NAME was used as the positive reference. Data obtained from three independent experiments are expressed as mean ± SEM. $^{\#\#}p < 0.01$ when compared with LPS-untreated cells, $^{*}p < 0.05$ and $^{**}p < 0.01$ when compared with the non-treated cells.

required for metal ion chelation [32]. Multiple blood transfusions cause secondary iron overload in thalassemia. The patients suffer from this complication, especially oxidative stress [6,7]. The results suggest that brazilin is an effective iron chelator *in vitro*. It should be further studied for iron toxicity alleviation in iron-loaded thalassemic mice for use as a therapeutic agent.

The properties of an antioxidant compound are involved in various mechanisms, including the prevention of chain initiation, binding of the transition metal ion, decomposition of peroxides, scavenging of free radicals, and hydrogen abstraction [18]. The structures of flavonoids and their derivatives show that antioxidant activity depends on the number and position of hydroxyl groups bound to the aromatic ring, which can be easily oxidized [42]. Brazilin exhibited DPPH radical scavenging activity and $ABTS^{\bullet+}$ radical scavenging activity. These results indicate that brazilin has a potential antioxidant activity. Therefore, a potential antioxidant should be able to scavenge or quench free radicals [43]. Moreover, the reducing capacity of polyphenols is an indicator of antioxidant activity [44], and the ability to reduce ferric ions to ferrous ions reflects the antioxidant properties of brazilin.

Oxidative stress is the production of more ROS than the capacity of antioxidant systems [45]. ROS includes hydroxyl radical ($OH^{\bullet}$), superoxide ($O_2^{\bullet-}$), and nitric oxide (NO). ROS maintains normal cellular physiological functions, but overproduction of ROS can cause cellular oxidative damage [46,47] leading to chronic organ diseases, such as liver fibrosis and cirrhosis [48]. An examination of ROS inhibition in the presence of brazilin in H_2O_2 stimulated ROS production in Huh7 cells indicated that brazilin inhibited ROS generation in hepatocytes. Thus, brazilin effectively protected hepatocytes against ROS. Under pathological conditions, NO production increases and contributes to cytotoxicity and tissue damage [12]. The present study demonstrated that brazilin inhibited NO production in LPS-stimulated Raw 264.7 cells in a dose-dependent manner, and this effect was shown without cytotoxicity. Brazilin has a poly-hydroxyl group, which can oxidize and scavenge free radicals. A subsequent study revealed that the inhibitory effect of brazilin on NO production was related to TNF-α and IL-6 gene expression as assessed *via* RT-qPCR. Brazilin attenuated the expression of IL-6 mRNA in LPS-stimulated RAW 264.7 cells in a dose-dependent manner. Treatment with 16 μM brazilin significantly decreased TNF-α mRNA levels in LPS-stimulated cells. It is known that pro-inflammatory cytokines, such as TNF-α and IL-6, are produced from macrophage cells during the inflammatory response [49]. Brazilin inhibits $NO^{\bullet}$ production and inducible nitric oxide synthase expression [50]. These results suggest that brazilin inhibits NO production and suppresses TNF-α and IL-6 mRNA expression in LPS-stimulated RAW 264.7 cells.

These results indicate that brazilin possesses antioxidant properties associated with iron chelation. In addition, brazilin directly inhibited ROS production. These properties are important for the treatment and prevention of oxidative stress-related diseases. Moreover, it shows anti-inflammatory activity *via* suppressed TNF-α and IL-6 mRNA expression in macrophage cells.

5. Conclusions

Brazilin is a bioactive compound in *C. sappan* that has iron chelation properties with a bidentate chelator and a stoichiometry of 2:1 complexation (brazilin: ferric ion). Brazilin exhibits antioxidant activity and inhibits ROS generation in hepatocytes. Moreover, brazilin can inhibit NO production, TNF-α, and IL-6 mRNA expression in LPS-stimulated RAW 264.7 cells. These findings suggest that the iron-binding, antioxidant, and anti-inflammatory activities of brazilin may alleviate oxidative stress in iron overload conditions, such as thalassemia, Alzheimer's disease, liver cirrhosis, and inflammatory disorders. This potential could lead to new therapeutic approaches, inspiring further research and development.

Ethical approval and consent to participate

This study did not involve any human and animal samples.

Data availability statement

Data included in article/supp. material/referenced in article.

CRediT authorship contribution statement

Somjintana Taveepanich: Writing – review & editing, Writing – original draft, Methodology, Investigation. **Kampanart Chayajarus:** Writing – review & editing, Conceptualization. **Jutharat Jittimanee:** Investigation, Formal analysis. **Naruedon Phusri:** Investigation, Formal analysis. **Paptawan Thongdee:** Investigation, Formal analysis. **Khemmisara Sawatdee:** Investigation, Formal analysis. **Pharit Kamsri:** Writing – original draft, Methodology, Formal analysis. **Auradee Punkvang:** Writing – original draft, Methodology, Formal analysis. **Khomson Suttisintong:** Writing – review & editing, Methodology, Formal analysis. **Pornpan Pungpo:** Writing – original draft, Methodology, Conceptualization. **Wanwisa Suwannaloet:** Investigation, Formal analysis. **Ruttiya Thongrung:** Writing – review & editing, Formal analysis. **Kanjana Pangjit:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e38213.

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