



Anti-ferroptotic lignan glycosides from *Zanthoxylum avicennae*: Isolation, structural characterization, and mechanistic evaluation

Loc Xuan Le¹, Truc Ngoc-Thanh Le¹, Thao Phuong Dao¹, Quan Hoang Nguyen², Thuy Nguyen-Bien Bui^{1*}

¹University of Health Sciences, Vietnam National University, Ho Chi Minh City, Vietnam

²Pasteur Institute, Ho Chi Minh City, Vietnam

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ABSTRACT

Introduction: Ferroptosis, a regulated cell death driven by iron-dependent lipid peroxidation, has been implicated in various liver diseases. Small molecules that counteract ferroptosis remain largely restricted to a few synthetic scaffolds, leaving natural products an underexplored source. *Zanthoxylum avicennae* (Lam.) DC. (Rutaceae) is used against inflammatory and hepatic conditions in Vietnamese traditional medicine. It contains diverse lignans and flavonoids capable of hydrogen-atom-transfer radical scavenging, the mechanism shared by established ferroptosis inhibitors. Its metabolites are therefore potential ferroptosis inhibitors. This study aimed to isolate, structurally characterize, and evaluate the anti-ferroptotic activity of its metabolites.

Methods: Metabolites were isolated from the leaves of *Z. avicennae* by 80% ethanol reflux extraction, solvent partitioning and silica gel column chromatography. Structures were elucidated by ¹H and ¹³C NMR spectroscopy. Anti-ferroptotic activity was assessed in an optimized RSL3-triggered ferroptosis model in HepG2 cells, using the MTT assay. The most active compound was evaluated for anti-lipid peroxidation activity using BODIPY 581/591 C11, labile iron pool modulation by FerroOrange, radical scavenging and antioxidant capacity using the DPPH, the cell-free BODIPY 581/591 C11, and the FRAP assay.

Results: (–)-isolariciresinol 3- α -O- β -D-glucopyranoside (compound **9**), a lignan glycoside, showed anti-ferroptotic activity ($EC_{50} = 5.89 \mu M$) without detectable cytotoxicity at 30 μM . It suppressed lipid peroxidation but did not reduce the intracellular labile iron. Compound **9** quenched DPPH and AAPH-derived peroxy radicals and displayed Fe³⁺-reducing capacity.

Conclusion: Compound **9** from *Z. avicennae* is an initial hit possessing radical-scavenging-associated protection in an RSL3-induced ferroptosis model.

Implication for health policy/practice/research/medical education:

A lignan glycoside, (–)-isolariciresinol 3- α -O- β -D-glucopyranoside, isolated from *Zanthoxylum avicennae* exhibited anti-ferroptotic, anti-lipid peroxidation, and antioxidant activities. This initial discovery provides a starting point for further mechanistic validation, structure-activity optimization, and in vivo evaluation in ferroptosis-driven disease models.

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Introduction

Zanthoxylum avicennae (Lam.) DC. is a deciduous plant of the Rutaceae family, found mainly across northern and central Vietnam and in parts of Africa and China. Traditionally, its leaves, roots, fruits, and bark have been

used against ailments including rheumatism, hepatitis, and wounds (1,2). *Z. avicennae* is rich in alkaloids, lignans, and flavonoids. What makes it particularly promising is not just the presence of these classes, but their diversity and synergistic potential, which is often broader than

*Corresponding author: Thuy Nguyen-Bien Bui,
Email: bnbthuy@uhsvnu.edu.vn

in many single-compound-dominant medicinal plants. Compared with many plants that are only traditionally cited, *Z. avicennae* has experimentally supported anti-inflammatory, antimicrobial, antioxidant, and possibly anticancer activities (3-6).

Ferroptosis, a recently defined form of regulated cell death, arises when iron-dependent oxidation drives phospholipid hydroperoxides to accumulate within cellular membranes until membrane integrity collapses (7,8). Uncontrolled peroxidation of membrane phospholipids enriched with polyunsaturated fatty acids (PUFAs) is the primary mechanism of ferroptosis execution, generated by both non-enzymatic Fenton-driven free-radical reaction and enzymatic lipoxygenase activity (9,10). Several interrelated antioxidant systems comprise the main cellular defense mechanisms that prevent ferroptotic-related cell death. The glutathione peroxidase 4 (GPX4)–glutathione (GSH) axis is one of the most important by directly converting phospholipid hydroperoxides to their corresponding non-toxic lipid alcohols, limiting the build-up of deadly species that damage membranes (10). Ferroptosis suppressor protein 1 (FSP1) simultaneously mediates a different GPX4-independent protective pathway by regenerating reduced coenzyme Q10 (CoQ10), a lipophilic antioxidant that stops lipid peroxidation chain reactions and intercepts lipid peroxy radicals within cellular membranes (10). Apart from these enzymatic systems, a wide range of antioxidant and cytoprotective genes, such as those involved in glutathione biosynthesis, iron homeostasis, and reactive oxygen species detoxification, are induced by the nuclear factor erythroid 2–related factor 2 (NRF2)-dependent transcriptional network (8). Pharmacological collapse of these defences, drives ferroptotic cell death, leading to the pathology of neurodegenerative disorders, ischemia–reperfusion injury in cardiac, hepatic, and renal tissues, as well as acute organ failure (11,12).

Given these characteristics, there is a pressing need for effective ferroptosis inhibitors that can protect from peroxidative damage. Ferrostatin-1 (Fer-1) and liprostatin-1 (Lip-1), two synthetic compounds that function as strong radical-trapping antioxidants without chelating iron, were discovered as a result (13). However, both compounds possess poor pharmacokinetic properties, limiting their preclinical translation (14,15). Natural products offer a chemically diverse and underexplored space for ferroptosis modulator discovery; baicalein, kaempferol, quercetin, and curcumin have all been reported as ferroptosis inhibitors (16-18), and lignan-class compounds, although less frequently studied, are emerging as a promising scaffold (19-21).

Despite this rationale, few studies have evaluated the anti-ferroptotic potential of compounds isolated from *Z. avicennae*. This study, therefore, aimed to (i) isolate and structurally characterize secondary metabolites from the leaves of *Z. avicennae*, (ii) screen anti-ferroptotic

activities in an optimized in vitro model in HepG2 cells, (iii) evaluate labile iron modulation activity and anti-lipid peroxidation capacity in HepaRG cells, and determine the radical scavenging and reducing capacity of the most active hit.

Materials and Methods

Materials

The leaves of *Z. avicennae* were collected in December 2024 at Son Tra, Da Nang, Vietnam. A voucher specimen (ZA-202412) was deposited at the University of Health Sciences, Vietnam National University, Ho Chi Minh city, after authentication.

Extraction and isolation process

The extraction and isolation process followed a published procedure (22). Dried *Z. avicennae* leaves (4.0 kg) underwent triplicate reflux extraction in 80% ethanol (40 L) at 50 °C for 24 hours. The pooled extracts were concentrated at 60-100 mbar. The crude residue was then resuspended in water and subjected to liquid–liquid partitioning. An initial extraction with *n*-hexane was performed to eliminate chlorophyll, lipophilic pigments, and other nonpolar impurities. The remaining aqueous phase was subsequently partitioned with ethyl acetate and *n*-butanol to obtain fractions of various polarities.

The ethyl acetate fraction (50 g) was chromatographed over silica gel (40-75 mesh, Fuji Silysia Chemical Ltd., Japan) using *n*-hexane-chloroform-methanol, with the solvent composition gradually adjusted from 10:0:0 to 0:8:2 (v/v/v), affording thirteen fractions (ZA-A1–ZA-A13). The *n*-butanol fraction (10 g) was chromatographed over silica gel (40–75 mesh) using chloroform-ethyl acetate-methanol, with the solvent composition gradually adjusted from 10:0:0 to 0:8:2 (v/v/v), yielding 10 fractions (ZA-B1–ZA-B10). All fractions were analyzed by thin-layer chromatography (TLC). Among them, fractions ZA-A2, ZA-A6, ZA-B4, ZA-B6, ZA-B7, and ZA-B9 exhibited more defined and well-resolved spots, indicating the presence of potentially pure or structurally distinct constituents. Selected fractions were further separated by silica gel (40–75 mesh) open-column chromatography using a gradient elution method of *n*-hexane–ethyl acetate ranging from 10:0 to 5:5 (v/v) to obtain compounds **1** (12.7 mg), **2** (296.0 mg), and **3** (67.5 mg) from 5.0 g of fraction ZA-2; compounds **4** (189.2 mg) and **5** (21.0 mg) from 1.0 g of fraction ZA-6. Compounds **6** (2.2 mg), **7** (5.6 mg), **8** (7.2 mg), and **9** (12.3 mg) were precipitated by repeated methanol washing from fractions ZA-B4, ZA-B6, ZA-B7, and ZA-B9, respectively.

NMR spectroscopic analysis and chromatographic conditions

Metabolites' structures were elucidated using NMR spectroscopy on a Bruker ARX-500 spectrometer. The residual solvent resonances, DMSO-*d*₆ (δ_{H} 2.50 and δ_{C}

39.6 ppm) and CD₃OD (δ_{H} 3.31 and δ_{C} 49.0 ppm), were used as internal standards for chemical shift calibration while recording spectra in DMSO-*d*₆ or CD₃OD. Scalar coupling constants (J) are indicated in hertz (Hz), whereas chemical shifts (δ) are shown in parts per million (ppm) in relation to tetramethylsilane (TMS). The resulting NMR data were analyzed and compared with previously reported spectral data to confirm the chemical structures of the purified metabolites.

Chromatographic purification was conducted by open-column chromatography utilizing a silica gel (40–75 mesh). The separation process was monitored by TLC on precoated silica gel 60 F₂₅₄ plates (Merck, Germany) under ultraviolet light at wavelengths of 254 and 365 nm.

Cell culture

HepG2 cells (ATCC HB-8065, USA), sensitive to RSL3 and suitable for high-throughput MTT-based readouts, were cultivated in DMEM (Thermo Fisher, Singapore) containing 10% fetal bovine serum (FBS; Sigma-Aldrich, Singapore). HepaRG cells (HPRGC10, Gibco, USA), possessing lipid metabolism and iron regulatory machinery, were used for lipid peroxidation evaluation and labile iron detection. HepaRG cells were maintained in William's E medium (Sigma-Aldrich, Singapore) containing 10% FBS, 5 µg/mL human insulin solution, 2 mM L-glutamine, and 50 µM hydrocortisone (Sigma-Aldrich, Singapore). Cells were cultivated at 37 °C in 5% CO₂ and subcultured when they reached approximately 70% confluence.

Metabolic activity assessment by MTT assay

HepG2 cells were seeded at densities of 2,500, 6,250, or 10,000 cells per well overnight. The following day, RAS-Selective Lethal 3 (RSL3; MedChemExpress LLC, USA) was added to induce ferroptotic cell death at final concentrations of 0.125, 0.25, 0.5, 0.75, 1, or 2 µM. After 24, 48, or 72 hours, cell metabolic activity was assessed by MTT assay.

To screen for compounds active at low-micromolar concentrations, HepG2 cells (6,250 cells per well) were treated with either 0.5 µM RSL3 alone or combined with 3 µM compounds for 24 hours. To evaluate the compound's intrinsic cytotoxicity, HepG2 cells were exposed to the compounds at 30 µM for 24 hours. DMSO was maintained at 0.5% across all conditions. DMSO (0.5%) and Lip-1 (0.1 µM; MedChemExpress, USA) served as vehicle and positive controls, respectively. After 24 hours, 10 µL of 5 mg/mL MTT (Sigma-Aldrich, Germany) was added, and cells were incubated for 3 hours. Formazan crystals were solubilized in 100 µL of acidified isopropanol. Optical density was measured at 570 nm on a Multiskan SkyHigh Microplate Spectrophotometer (Thermo Fisher, Singapore) (23).

Lipid peroxidation evaluation by BODIPY C11 staining

HepaRG cells (20,000 cells per well in ibidi µ-slide 8-well) were pretreated for 15 minutes with 0.5% DMSO, 0.1 µM Lip-1, or 45 µM compound **9**, and subsequently challenged with 1 µM RSL3 for 2 hours. Cells were stained with 2 µM BODIPY C11 (Thermo Fisher, Singapore) to detect cellular lipid peroxidation and counterstained with Hoechst 33342 (1 µM; Thermo Fisher, Singapore) to label nuclei. After 30 minutes, cells were washed twice with 1× HBSS (Thermo Fisher, Singapore) and imaged using a ZEISS Axiovert 5 fluorescence microscope (Zeiss, Germany) with the FITC and DAPI filters.

Labile iron detection by FerroOrange

HepaRG cells (20,000 cells/well in an ibidi µ-slide 8-well) were treated with (NH₄)₂Fe(SO₄)₂ (FAS; Sigma-Aldrich, Singapore) at 100 µM alone or in combination with 45 µM compound **9**, 0.5% DMSO, or 100 µM 2,2'-bipyridine (BPy, Sigma-Aldrich, Singapore). After 60 minutes, cells were washed and incubated with 200 µL of 1× HBSS containing 1 µM FerroOrange (Sigma-Aldrich, Singapore) for 30 min. Cells were washed twice with 1× HBSS and imaged by a ZEISS Axiovert 5 fluorescence microscope using the TRITC filter.

Cell-free BODIPY C11 assay

The assay was performed following Tschuck et al (23). 30 µL of compound **9** (final concentrations of 2, 10, 20, and 40 µM) was mixed with 30 µL of 2 µM BODIPY C11 and 30 µL of 7.5 mM AAPH (Sigma-Aldrich, Singapore). The mixture was maintained at 42 °C for 1 hour on a SpectraMax Mini Multi-Mode Microplate Reader (Molecular Devices, USA) to generate AAPH-derived peroxy radicals. Fluorescence intensity of oxidized BODIPY C11 was measured at $\lambda_{\text{ex}}/\lambda_{\text{em}} = 480/525$ nm.

DPPH assay

A mixture of 50 µL of 50 µM DPPH (Sigma-Aldrich, Singapore) and 50 µL of compound **9** (final concentrations of 2, 2.5, 10, 20, 30, and 45 µM) was incubated in the dark at room temperature for 15 min. Absorbance was recorded at 517 nm on a Multiskan SkyHigh Microplate Spectrophotometer. DMSO and ascorbic acid (Sigma-Aldrich, Singapore) served as vehicle and positive controls, respectively (23).

Ferric reducing antioxidant power (FRAP) assay

A mixture of 8 µL of 10 mM TPTZ (Sigma-Aldrich, Singapore) in 40 mM HCl, 80 µL of 300 mM sodium acetate buffer pH 3.6, and 8 µL of 20 mM FeCl₃, and 5 µL of compound **9** (final concentrations of 2 and 10 µM) was kept at room temperature for 15 min. Optical density was measured at 593 nm on a Multiskan SkyHigh Microplate Spectrophotometer. Antioxidant activity was determined by interpolation from a Trolox calibration curve and

reported as μM Trolox equivalents. DMSO and ascorbic acid served as vehicle and positive controls, respectively (24).

Data analysis and statistical methods

All graph data were displayed as individual values, mean, or mean and individual values $\pm$ SEM from three independent experiments. Statistical analysis used one-way ANOVA followed by Dunnett's multi-group comparisons test against the RSL3 or vehicle control DMSO group, or a two-tailed paired student's *t* test for two-group comparisons. $P < 0.05$ indicates statistical significance. Figures were generated using GraphPad Prism 10.0. Images of cells were acquired under identical exposure and display settings, and were processed using Zeiss ZEN 3.7.

Results

Structure investigation of metabolites isolated from the ethanolic leaf extract of *Z. avicennae*

Nine compounds were isolated from 80% ethanol extract of the leaves of *Z. avicennae* and identified by NMR spectroscopy in comparison with literature data: the coumarin 8-methoxyanisocoumarin H (1) (25); the furoquinoline alkaloids skimmianin (2) (26) and γ -fagarin (3) (27); the 2-alkyl-4-quinolone alkaloids 2-*n*-pentyl-1-methylquinolin-4(1H)-one (4) (28) and 2-*n*-heptyl-1-methylquinolin-4(1H)-one (5) (26); the flavonoid glycoside hesperidin (6) (29); the propiophenone glycoside 2,6-dimethoxy-4-propionylphenyl O- $[\alpha$ -L-rhamnopyranosyl(1 $\rightarrow$ 6)]- β -D-glucopyranoside (7) (30); and the isolariciresinol lignan glycosides

(+)-isolariciresinol 3- α -O- β -D-glucopyranoside (8) (31) and (-)-isolariciresinol 3- α -O- β -D-glucopyranoside (9) (32) (Figure 1). NMR spectroscopic data are provided in Supplementary file 1.

Optimization of the *in vitro* RSL3-induced ferroptosis model

Prior to evaluating the anti-ferroptotic activity of the metabolites isolated from *Z. avicennae* leaves extract, a cell-based screening model was optimized. As shown in Figure 2A, cell viability increased with cell density across all conditions. Cultures at 2,500 cells per well showed low cell viability regardless of RSL3 concentration or exposure time. In contrast, 10,000 cells per well exhibited RSL3 resistance at 24 and 48 hours. The intermediate density, 6,250 cells per well, yielded cell viability that was dependent on RSL3 concentration and exposure time. Treatment duration had minimal effect on cell viability. Optimal screening conditions were therefore set at 6,250 cells/well, 0.5 μM RSL3 for 24 hours.

Anti-ferroptotic screening and cytotoxicity of compounds 1–9

Nine compounds isolated from *Z. avicennae*, including coumarin (1), furoquinoline alkaloids (2, 3), 2-alkyl-4-quinolone alkaloids (4, 5), flavonoid glycoside (6), propiophenone glycoside (7), and isolariciresinol lignan glycosides (8, 9), were evaluated for cytotoxicity on HepG2 cells. Compounds 1, 4, 5, 6 at 30 μM reduced HepG2 cell viability by 24–46% relative to vehicle, while the remaining compounds (compounds 2, 3, 7, 8, and 9) did not produce a statistically significant cytotoxic effect

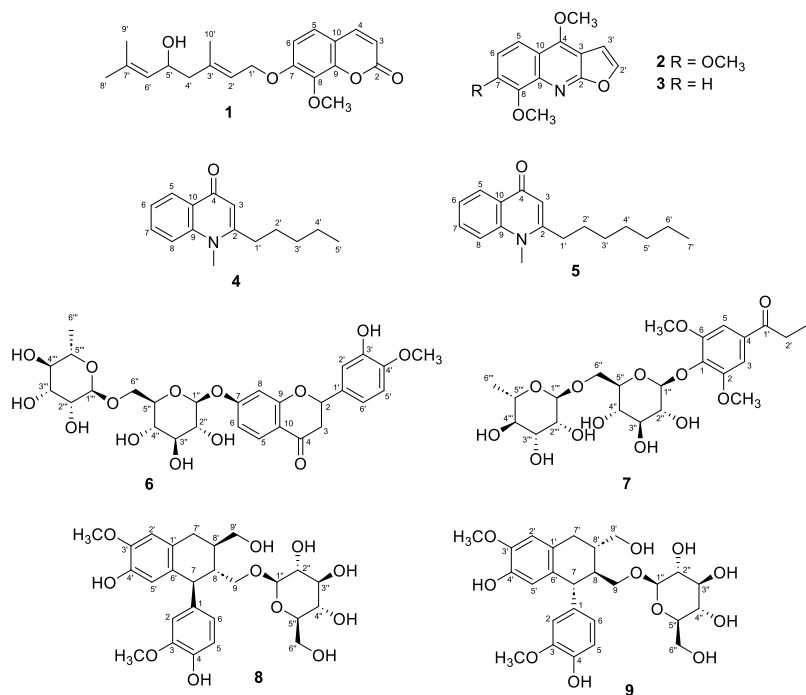


Figure 1. Chemical structures of compounds 1–9 isolated from the ethanolic leaves extract of *Zanthoxylum avicennae*.

(Figure 2B).

As shown in Figure 2C, RSL3 reduced HepG2 cell viability to 35.3% relative to vehicle control. Compounds 1–7 failed to protect cells against RSL3, whereas compounds 8 and 9 preserved cell viability at 55% and 56%, respectively. Since compounds 8 and 9 were enantiomers with comparable cytotoxicity profiles and screening responses, compound 9 (Figure 2D) was selected as a representative for further mechanistic characterization.

Dose-dependent anti-ferroptotic effect of compound 9

EC₅₀ of compound 9 was determined to be 5.89 μ M (Figure 2E). Lip-1 at 0.1 μ M protected cells against RSL3, with cell viability comparable to that of the vehicle (108.9%). At 0.94 and 1.88 μ M, compound 9 showed no detectable protection. The protective effect of compound 9 emerged at 3.75 and 7.5 μ M (cell viability 58.8% and 74.3%, respectively) and reached a plateau at 15 and 45 μ M (cell viability 95.7% and 107.3%, respectively). Thus, the protective effect of compound 9 was dose-dependent within the tested range.

Compound 9 reduced RSL3-induced lipid peroxidation in cells

BODIPY C11 staining was employed to determine whether compound 9 could inhibit lipid peroxidation in HepaRG

cells. RSL3 (1 μ M) produced a robust increase in green fluorescence across the cell population, consistent with extensive ferroptotic lipid peroxidation (Figure 3), while Lip-1 (1 μ M) markedly suppressed RSL3-induced lipid peroxidation. Compound 9 (45 μ M) visibly attenuated the green fluorescence relative to RSL3 alone, though the residual signal remained above that of the Lip-1 group. These observations indicate that compound 9 suppresses RSL3-induced lipid peroxidation in intact cells.

Compound 9 did not reduce intracellular labile iron

FerroOrange staining was employed to determine whether compound 9 inhibit ferroptosis by reducing labile iron. When HepaRG cells were spiked with 100 μ M FAS, orange fluorescence markedly increased compared with vehicle-treated cells. Iron chelator Bpy (100 μ M) reduced the labile iron levels, whereas compound 9 (45 μ M) did not (Figure 4). Thus, the mechanism action of compound 9 unlikely to involve labile iron alteration.

Compound 9 as a potent antioxidant

AAPH thermally generates peroxy radicals that oxidize the polyunsaturated linker of BODIPY C11. AAPH increased the green fluorescence intensity up to three-fold compared to vehicle (Figure 5A), and Lip-1 at 2 μ M inhibited the oxidation reaction. Compound 9 inhibited

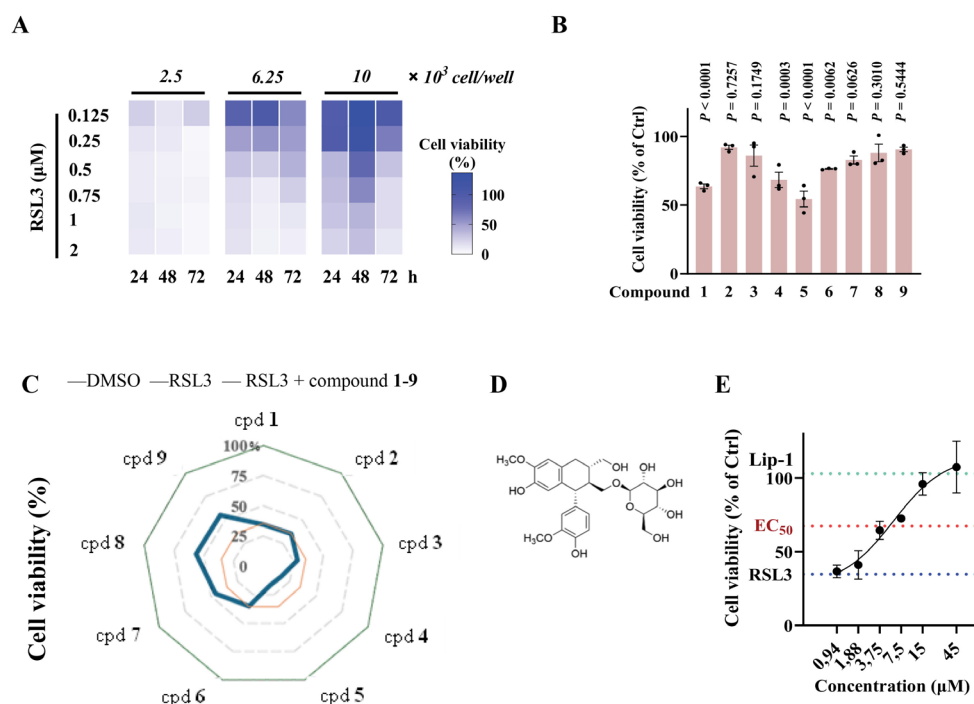


Figure 2. Screening for *in vitro* anti-ferroptotic activity of compounds 1–9 isolated from ethanolic leaves extract of *Zanthoxylum avicennae*. (A) Optimization of the *in vitro* RSL3-induced ferroptosis model in HepG2 cells (cell density $\times$ RSL3 concentration $\times$ exposure time), assessed by MTT assay. (B) Cytotoxicity of compounds 1–9 at 30 μ M in HepG2 cells (24 h), assessed by MTT assay. (C) Anti-ferroptotic activity of compounds (cpd) 1–9 at 3 μ M in HepG2 cells against RSL3 (0.5 μ M) for 24 h. (D) Structure of compound 9 [(–)-isolaricresinol 3- α -O- β -D-glucopyranoside]. (E) Dose–response anti-ferroptotic activity of compound 9 against RSL3 (0.5 μ M) in HepG2 cells for 24 h. Lip-1: Liprostatin-1 (positive control, 0.1 μ M); DMSO: vehicle control (0.5%). Data are individual values (A), individual values and mean $\pm$ SEM (B), mean (C) or mean $\pm$ SEM (D) from three independent experiments. Statistical analysis was performed using one-way ANOVA with Dunnett's multiple comparisons against the DMSO vehicle control (B).

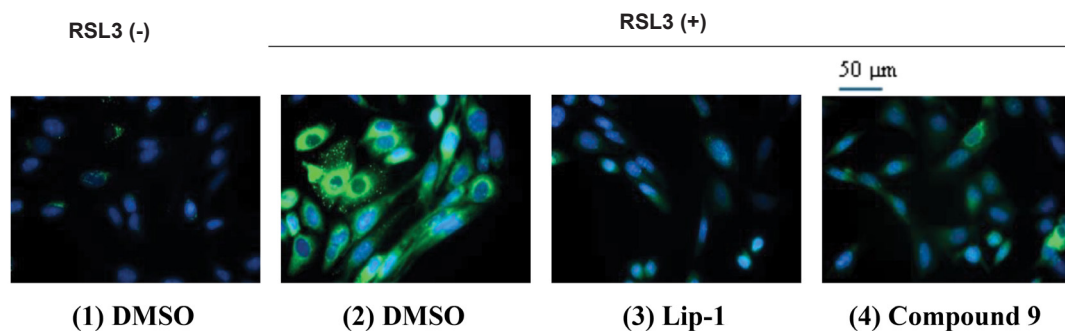


Figure 3. Compound **9** reduced RSL3-induced lipid peroxidation in HepaRG cells. Representative fluorescence microscopy images (40× magnification) from three independent experiments. HepaRG cells were pretreated for 15 min with: (1, 2) 0.25% DMSO; (3) 0.1 μ M Liproxstatin-1 (Lip-1, positive control); or (4) 45 μ M compound **9**, followed by treatment with (1) 0.25% DMSO or (2, 3, 4) 1 μ M RSL3 for 2 h. Cells were stained with 2 μ M BODIPY C11 (green, oxidized or peroxidized lipids) and counterstained with Hoechst 33342 (blue, nuclei). Scale bar, 50 μ m.

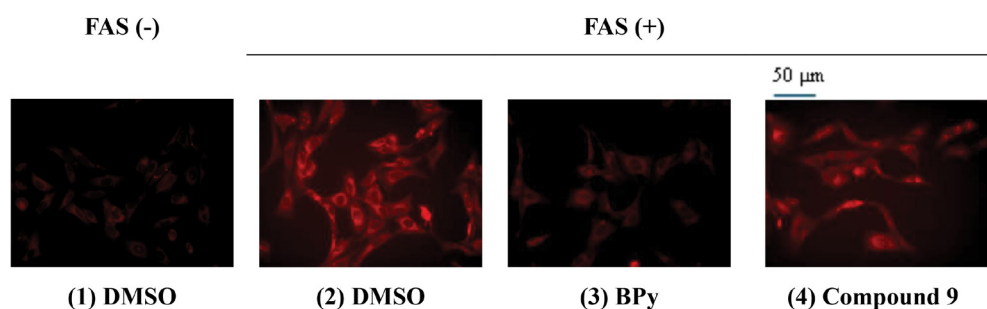


Figure 4. Compound **9** did not reduce intracellular labile iron in HepaRG cells. Representative fluorescence microscopy images (40× magnification) from three independent biological experiments. HepaRG cells were treated with (1) 0.5% DMSO, ferrous ammonium sulphate (FAS, 100 μ M) in combination with (2) 0.5% DMSO, (3) 100 μ M 2,2'-bipyridine (Bpy), or (4) 45 μ M compound **9**. Cells were stained with FerroOrange (1 μ M, 30 min). Scale bar: 50 μ m.

the radicals in a dose-dependent manner, although less effectively than Lip-1. At 2 and 10 μ M, compound **9** did not statistically reduce the fluorescence intensity. However, at 20 and 40 μ M, radical-trapping activity was apparent, compared to the AAPH-treatment alone group (127% and 115%, respectively, $P < 0.0001$).

DPPH radicals decolorize in the presence of radical scavengers. Ascorbic acid produced a strong scavenging response ($IC_{50} = 5.15 \mu$ M) while compound **9** was less potent. Compound **9** neutralized 43% and 77% DPPH radicals at 20 μ M and 45 μ M, respectively (Figure 5B).

The FRAP assay measures total reducing capacity by converting the ferric-TPTZ complex to the ferrous form under acidic conditions. Compound **9** showed lower activity than ascorbic acid at 2 μ M (10.07 vs. 12.88 μ M Trolox equivalents, $P = 0.0003$) but exceeded it at 10 μ M (95.83 vs. 39.84 μ M Trolox equivalents, $P < 0.0001$) (Figure 5C).

In summary, compound **9** exhibits radical-scavenging and antioxidant activity in cell-free systems.

Discussion

Screening of natural products as ferroptosis modulators has attracted increasing attention, given the involvement of ferroptosis as a driver of multiple diseases related

to excessive cell death (12,33). Natural products offer a chemically diverse, underexplored space for the discovery of selective inhibitors with potentially improved bioavailability. Within this space, lignans are an under-investigated chemical class. Among the nine *Z. avicennae* compounds tested, the lignan glycoside compound **9** was identified as the most active compound with moderate anti-ferroptotic effect against RSL3 in HepG2 cells with negligible cytotoxicity up to 30 μ M. Its EC_{50} of 5.89 μ M places compound **9** in the same potency as nigratine (34), bisbenzylisoquinoline alkaloids (35), and baicalein (16), while it is less potent than synthetic radical-trapping antioxidants such as Lip-1, which acts at nanomolar to low-micromolar concentrations in most models (36). Lip-1 carries a diarylamine moiety that traps lipid peroxyl radicals via N-H hydrogen-atom transfer; compound **9**, by contrast, carries two free phenolic groups, which may exhibit, though less efficiently, radical-trapping activity via hydrogen-atom transfer (37). From a structure-activity perspective, the panel of *Z. avicennae* compounds tested is broadly consistent with the hypothesis that free phenolic hydroxyl groups capable of hydrogen-atom transfer underpin the observed activity. The coumarin (compound **1**) and the 4-quinolone and furoquinoline alkaloids (compounds **2–5**) lack free phenolic groups

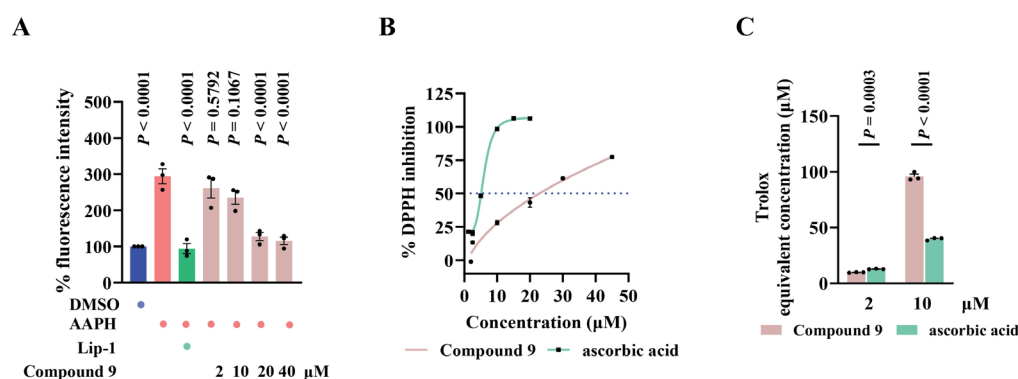


Figure 5. Radical-scavenging and reducing capacity of compound 9. (A) Cell-free BODIPY C11 assay using AAPH (7.5 mM) as a thermal peroxy-radical generator, co-treated with compound 9, DMSO (0.5%), or Liproxstatin-1 (Lip-1, 2 μM). (B) DPPH radical-scavenging assay of compound 9 (2–45 μM). (C) FRAP antioxidant activity of compound 9 (2 and 10 μM) expressed as μM Trolox equivalents. Ascorbic acid (2 and 10 μM): positive control. Data are individual values and mean ± SEM (A, C) or mean ± SEM (B) from three independent experiments. Statistical analysis was performed using one-way ANOVA with Dunnett's multiple comparisons against the AAPH-treated group (A) or two-tailed paired Student's *t*-test (C).

and showed no protection. Unlike the flavonoid glycoside hesperidin (compound 6) and the propiophenone glycoside (compound 7), which carry glycosylated or methylated phenolic positions, the isolariciresinol lignan glycosides (compound 8) and (compound 9) retain two free phenolic groups on their core, showing the strongest protective effect. Importantly, compound 9 also suppressed RSL3-induced lipid peroxidation in intact HepaRG cells, providing direct cellular evidence that it counteracts ferroptosis by attenuating lipid peroxidation rather than acting only in a cell-free system or providing broad protection as a potent antioxidant. We propose that compound 9 may act as a hydrogen-atom-transfer radical-trapping antioxidant at lipid membranes, intercepting propagating peroxy radicals generated during RSL3-driven GPX4 inactivation, rather than by modulating the labile iron pool.

This study has several limitations. First, the BODIPY C11 and FerroOrange cell staining assays were qualitative. Quantitative analysis by flow cytometry should be employed to provide statistical confirmation. Oxidized phosphatidylethanolamine, a marker of ferroptosis events, can also be determined using UPLC-MS/MS. Second, whether compound 9 also acts on additional mechanisms, such as stabilizing GPX4, activating NRF2-dependent antioxidant programmes, or modulating the FSP1–CoQ₁₀ axis, remains unknown. Lastly, in vivo validation in established ferroptosis-associated disease models has not yet been performed, and the translational applicability of compound 9 therefore remains to be determined.

Conclusion

An optimized in vitro RSL3-induced ferroptosis model in HepG2 cells (6,250 cells/well, 24-h exposure, 0.5 μM RSL3) was established to screen nine secondary metabolites isolated from the leaves of *Z. avicennae*, comprising coumarin, alkaloid, flavonoid, phenolic

glycoside, and lignan-glycoside classes. Among the nine compounds, the lignan glycoside (–)-isolariciresinol 3- α -O- β -D-glucopyranoside (compound 9) exhibited moderate anti-ferroptotic activity ($EC_{50} = 5.89 \mu\text{M}$) with negligible cytotoxicity at 30 μM. The protective effect was associated with radical scavenging and attenuation of lipid peroxidation, rather than iron chelation. This initial discovery of compound 9 provides a structural starting point for further mechanistic validation, structure-activity optimization, and in vivo evaluation in ferroptosis-driven disease models.

Authors' contribution

Conceptualization: Thuy Nguyen-Bien Bui, Loc Xuan Le.

Data curation: Thuy Nguyen-Bien Bui, Loc Xuan Le.

Formal analysis: Thao Phuong Dao, Truc Ngoc Thanh Le, Thuy Nguyen-Bien Bui, Loc Xuan Le.

Funding acquisition: Thuy Nguyen-Bien Bui, Loc Xuan Le.

Investigation: Thao Phuong Dao, Truc Ngoc Thanh Le, Quan Hoang Nguyen.

Methodology: Loc Xuan Le, Thuy Nguyen-Bien Bui.

Project administration: Thuy Nguyen-Bien Bui, Loc Xuan Le.

Resources: Quan Hoang Nguyen, Loc Xuan Le.

Software: Loc Xuan Le.

Supervision: Quan Hoang Nguyen.

Validation: Quan Hoang Nguyen.

Visualization: Thao Phuong Dao, Truc Ngoc Thanh Le, Loc Xuan Le.

Writing—original draft: Thao Phuong Dao, Truc Ngoc Thanh Le, Loc Xuan Le.

Writing—review & editing: Thuy Nguyen-Bien Bui, Loc Xuan Le.

Conflict of interests

None to declare.

Declaration of AI-assisted tools in the writing procedure

The authors used Perplexity Pro and Anthropic Claude to assist with language polishing and literature review. AI-assisted output was thoroughly reviewed and verified by the authors.

Ethical considerations

Experiment protocols were approved by the University of Health Science, Vietnam National University, Ho Chi Minh City (C2023-44-08). All authors observed copying and plagiarism issues.

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Supplementary files

Supplementary file 1. ¹H-NMR and ¹³C-NMR Spectroscopic data of compounds isolated from *Z. avicennae*.

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