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Research Article

Xanthine Oxidase Inhibition and Anti-Lung Cancer Effects of the Lonicera japonica Thunb–Paris polyphylla var. chinensis Herbal Pair

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Abstract

In Vietnamese folk medicine *Lonicera japonica* Thunb. flowers (LTF) had recognized significantly attentions for gout treatment therapies, and LTF is frequently combined with the rhizomes of *Paris polyphylla* var. *chinensis* (PPR) for lung cancer remedy. This study evaluated *in vitro* therapeutic potential of this herbal pair. Their chemical structures were explicated by extensive spectroscopic analyses, covering MS and NMR spectra. The xanthine oxidase preventive effects of the isolated compounds and their extract mixtures, as well as their effects on A549 cell repressive capacity, were researched. Ferulic acid was identified for the first time from LTF together with chlorogenic acid, while quercetin and β-sitosterol-3-O-β-D-glucopyranoside were isolated from PPR. Mixture of PPR-MeOH and E50B (at ratio 1:1) restricted growth of A549 cancer cells with IC_{50} of 4.11±0.16 µg/mL. The LTF–PPR mixture also activated the apoptotic factors comprising caspase-3, caspase-9, and p53. The findings demonstrated for the first time the XO inhibitory capactity of PPR-MeOH and E50B are 42.12 ± 0.34 and 33.89±1.36, respectively. The present results recommended that applying LTF and PPR in gout and lung cancer treatment needs to insightly more research in the future.

Keywords: *Lonicera Japonica* Thunb, *Paris polyphylla*, *enzyme xanthine oxidase, anti-lung cancer.*

INTRODUCTION

The advancement of adjunctive therapeutic approaches for the management of gout has crucial clinical significance. In the body, xanthine oxidase (XO) is a critical enzyme catalyzing the oxidation of xanthine to uric acid which may cause hyperuricemia[1,2]. One of the basic therapeutic strategies in the limitation of uric acid overproduction in gout care and its related complications is that many recently synthesized antihyperuricemic drugs had been developed and invented. However, several clinically applied uric acid–lowering drugs elicited side effects and toxicity. Therefore, the study of useful natural products for the gout prevention and treatment have been highly required. Lung cancer evolves into a dominant factor of cancer-related mortality worldwide[3]. The modern therapy methods encompassing surgical resection, chemotherapy, radiotherapy and targeted therapies. However, these modalities are often connected with stringent side effects and non-responsiveness[4]. The natural medicinal products are promising applicants with a safe and effective anticancer potential as well as sustainable health protection. These products have been proven capacities of modulating censorious oncogenic pathways, activating apoptosis, angiogenesis inhibition, and immune surveillance boostion in compared with standard chemotherapeutics[5,6].

In family Caprifoliaceae, Lonicera japonica Thunb. is a valuable herb, and its flowers (LTF) are utilized in many traditional medicine remedies. One of the a mainly active chemical compounds attracting appreciable attention is chlorogenic acid. Advanced pharmacological investigations had illustrated that L. japonica has been exhibited to have benefit biological activities, such as antibacterial, anti-inflammatory, antiviral, anti-endotoxin, lipid-lowering, antipyretic, and other merit therapeutic effects[7]. Paris polyphylla var. chinensis, a rarely perennial herb, belongs to family Trilliaceae. Its rhizomes (PPR) have long been applied to cure inflammation, sore throat, snake envenomation, severe pain, and convulsive syndromes[8]. Many recently pharmacological researches disclosed that

phytochemical compounds isolated from the PPR and its crude extracts owned potential biological activities, for instance anticancer[9], antioxidant[10], antibacterial[11], hemostatic[12], and anthelmintic effects[13]. The PPR have been reported to strongly exhibit potential of cancer cell lines, for example CCRF-CEM leukemia cells, ECA109, CaEs-17, HL-60, HepG2, BGC-823, as well as LoVo, SW-116 and murine lung adenocarcinoma cells[14,15,16,17,18]. In Vietnamese folk medicine, the LTF have been widely applied for gout cure. Especially, the LTF was combined with PPR for lung cancer cure with verified clinical cases. However, the XO inhibitory potential of chlorogenic acid in the LTF has not been reported. Moreover, the XO and A549 cell growth inhibitory capacity of combination of two herbs are currently deficient in investigations. Some works have illustrated that herbal extracts, particularly in polyherbal formulations, supply superior therapeutic effects when compared with equivalent doses of single herbs administered alone, underscoring the significance of synergistic interactions in herbal medicine[19-21]. This work was conducted to evaluate the XO inhibitory and A549 cell growth proliferation activities of the herbal pair Paris polyphylla var. chinensis and Lonicera japonica Thunb., as well as their isolated chemical constituents.

MATERIALS AND METHODS

Chemicals, reagents and instruments

The chemicals and reagents comprised xanthine oxidase (XO), xanthine (>99%), allopurinol, chlorogenic acid, and ferulic acid. Organic solvents were used for extraction and chromatographic separation such as ethanol 95% (EtOH), methanol 95% (MeOH), ethyl acetate (EtOAc), dimethyl sulfoxide (DMSO), and methylene chloride (DCM). Additional reagents are phosphate buffer, Tris–HCl, NaCl, Tween 20, Tris Buffered Saline with Tween 20 (TBST), nonfat dry milk, sodium dihydrogen phosphate, disodium hydrogen phosphate, hydrochloric acid, and sodium hydroxide. Reagents for cell-based assays are Kaighn's modification of Ham's F-12 medium (KMH F-12), fetal bovine serum (FBS), phosphate-buffered saline (PBS), and penicillin–streptomycin. The MTT reagent [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] and other reagents. The main instruments are such as rotary evaporator; normal-phase silica gel, reversed-phase silica gel (RP-18), and Sephadex LH-20 for column chromatography; TLC plates (silica gel 60 F254 and RP-18 F254s); and high-performance liquid chromatography (HPLC) systems, along with other equipments.

Plant materials

The LTF and PPR of Paris polyphylla var. chinensis were gathered from Thai Nguyen and Lai Chau provinces, Vietnam. The plant materials were taxonomically authenticated by Dr. Quang Ung Le, in partnership with the Classification and Identification Committee of the Faculty of Agriculture Technology, Thai Nguyen University of Agriculture and Forestry. This committee has nine experts specializing in plant taxonomy, botany, pharmacognosy, and herbal sciences.

Extraction and isolation

The LTF in flowering stages (green bud stage-A, white bud and silver flowering stage-B and golden flowering stage-C) were washed by distilled water and dried in oven at 45 ^{o}C for 24 hours (hr). Five grams of 50-mesh sieved dry powder were separately extracted two times with EtOH at concentration of 50% (vol/vol) (E50A, E50B and E50C, respectively with flowering stages) at 30 ^{o}C by ultrasonic and then was filtered to give filtrate combinations and concentrated in a rotary evaporator at 45 ^{o}C. The dehydrated fractionation yield was calculated by using electronic scale, then dissolved in dimethyl sulforide (DMSO) to give a regular concentration to perform continue experiments

Two kilograms of PPR powder were exhaustively extracted with 15 L 95% MeOH by ultrasonic (three times, 90 min each at 40 °C). The combined MeOH extracts were condensed under reduced pressure to obtain a dark crude extract (PPR-MeOH extract 362.0 g). Then, this extract was suspended in distilled water (1 L) and successively partitioned with EtOAc to yield an EtOAc fraction (EtOAc, 82.6 g). The EtOAc extract was subjected to silica gel column chromatography using a gradient elution system of dichloromethane/methanol/water (from 100% DCM to MeOH/H_2O, 1/2/0.01, v/v) to give six fractions (PR-1→PR-6). Fraction PR-1 (0.6 g) was further purified by silica gel column chromatography eluted with DCM/MeOH (from 100% DCM to MeOH 10:1, v/v), gaining compound PR-1A, identified as β-sitosterol-3-O-β-D-glucopyranoside (44.3 mg). Fraction PR-4 (13.4 g) was separated by reversed-phase C18 column chromatography using n-hexane/ethyl acetate (5:1, v/v) as the eluent to obtain compound PR-4D (50.0 mg) chromatographed on a silicagel column to give compound PR-4D1 (quercetin, 22.0 mg). The separation procedures of compounds PR-1A and PR-4D1 are briefed in Figure 1

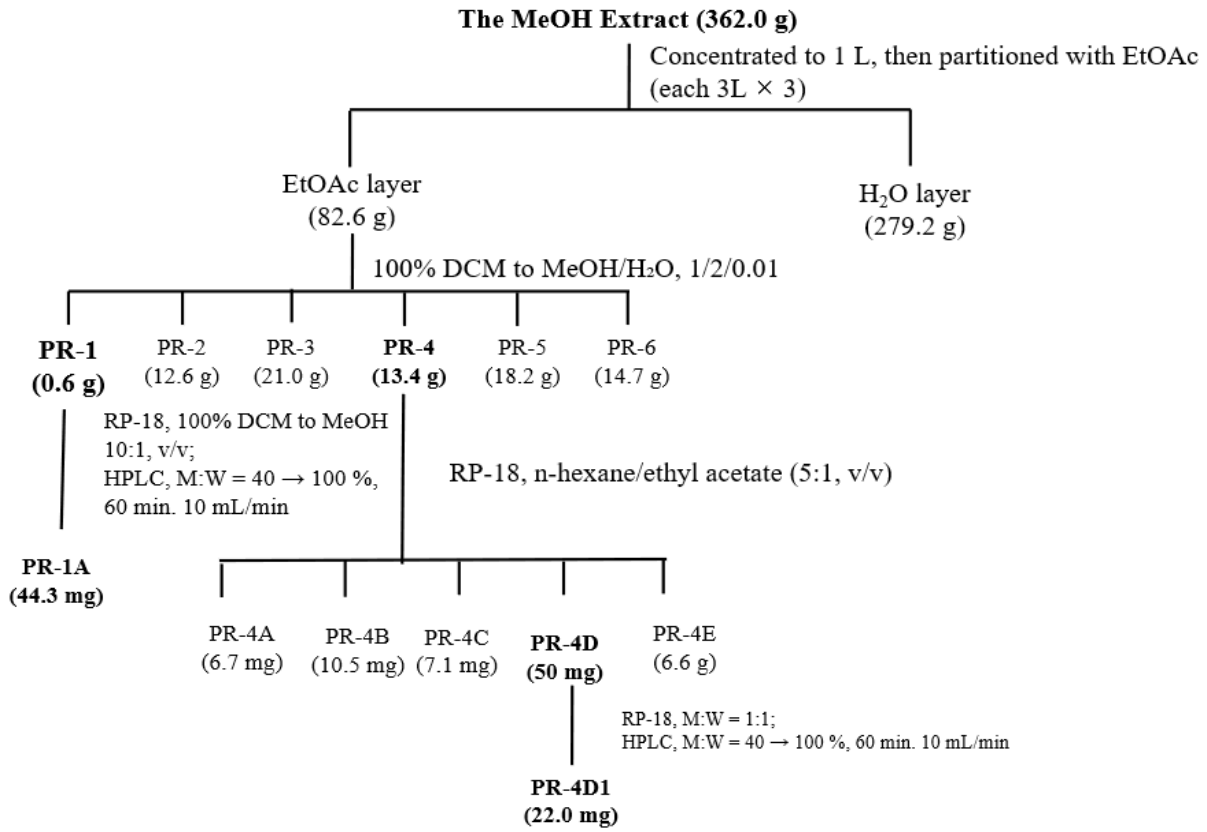

Figure 1. Schematic diagram of PR-1A and PR-4D1 isolation from ethyl acetate fraction

High performance liquid chromatography analysis

The phenolic constituents in the E50A, E50B and E50C extracts were quantitatively analyzed by HPLC. The system (Hitachi, Tokyo, Japan) was installed with a Chromaster 5110 pump, Chromaster 5210 autosampler, Chromaster 5430 diode array detector, and a Mightysil RP-18 GP column (4.6 × 250 mm; Kanto Chemical Co. Inc., Tokyo, Japan). The mobile phase comprised solvent A (distilled water) and solvent B (MeOH), both adjusted to pH 2.8 with H_3PO_4. The following gradient program was performed: 0-3 min, 5% B; 3-6 min, 7-10% B; 6-10 min, 10-15% B; 10-20 min, 15-20% B; 20-25 min, 20-25% B; 25-30 min, 25-28% B; 30-35 min, 28-30% B; 35-40 min, 30-40% B; 40-45 min, 40-42% B; 45-50 min, 42-45% B; 50-60 min, 45-30% A; and 60-65 min, 35-30% B. Chromatographic settings were performed as follows: flow rate of 1.0 mL/min, injection volume of 20 µL, and UV detection at 280 nm. The column temperature was continued at 40°C during the analysis. The phenolic constituents were resulted in by interpolation using the linear regression plot from the standard component solution.

Xanthine oxidase inhibition assay

The XO inhibitory experiments of compounds and extracts were performed according to Chen et al. (2010)[22]. Various concentrations of samples were prepared in 1% DMSO. The assay mixture comprised 40 µL of 1% DMSO (blank) or compound solution and 60 µL of XO enzyme solution (0.02 U/mL in 50 mM PBS, pH 7.5), freshly prepared before the experiment. After incubation at 37°C, the absorbance was determined at 295 nm after 45 min. The positive control compound is allopurinol. The XO inhibitory ability was quantified: inhibition (%) = $(A_0 - A_t)/A_0 \times 100\%$, in which A_0 and A_t correspond to the absorbance of the blank and the test sample, respectively. The IC_{50} value was calculated as the dosage required to inhibit XO by 50%.

Cytotoxic effects of compounds and fraction mixtures

Cell culture

A549 cells were cultured in KMH F-12 medium added with 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin. The culture medium was newed every 72 hr, and the cells were stably incubated in a 5% CO_2 and 37 °C condition incubator.

Cell Viability Assay

A549 cells were cultured in 96-well plates with a 5×10^3 density per well in 200 µL of culture medium, with each assay performed in three times. Following a 24 hr attachment period, the cells were exposed to compounds or extracts at the prepared dosages in serum-free medium for 48 hr. The positive control compound in this assay is fluorouracil, while cells treated with 0.2% DMSO functioned as the vehicle control. Cell viability was subsequently evaluated using

the MTT assay according to an earlier reported method[23].

Western blot analysis

The PPR-MeOH and E50B mixture (at ratio 1:1), presenting the strongest anticancer potential (Table 3), was used to induce apoptosis in A549 cells. Whole cell protein extracts were prepared using 1× RIPA buffer supplemented with a complete protease inhibitor cocktail. The centrifugation (12,000 × g for 20 minutes at 4 °C) was used to purify lysates to give the supernatants. Protein concentrations were quantified by the Bradford assay, the proteins mixing with 2×sample loading buffer were incubated at 95 °C for 5 min, and separated on 12% polyacrylamide gels using the Mini-Protean 3 electrophoresis system (Bio-Rad). Proteins were subsequently transferred onto nitrocellulose membranes which were then blocked with 5% non-fat milk/TBST (10 mM tris-HCl, pH 7.5, 150 mM NaCl, 0.1% tween 20) for 2 hr at room temperature and then cultured overnight at 4 °C with the relevant primary antibodies. Following washing with blocking buffer three times for 0.5 hr, the membranes were incubated for 2 hours with horseradish peroxidase–conjugated goat anti-mouse or anti-rabbit IgG secondary antibodies. After extensive washing thrice for 1 hr with Tris-buffered saline Tween 20, immunoreactive bands were detected by Enhanced chemiluminescence Detection Kit using a LAS-3000 luminescent imaging system. Antibody dilutions for Western blot analysis were as follows: β-actin (1:3000); caspase-3, caspase-9, and p53 (1:500), as formerly described[24].

Statistical analysis

The experimental data were analyzed using one-way analysis of variance (ANOVA) to evaluate statistically significant differences among treatment groups. Post-hoc comparisons were carried out using the least significant difference (LSD) test. All statistical procedures were performed with the SAS software package (SAS Institute, 1990), and significance was considered at $p < 0.05$.

RESULTS AND DISCUSSION

The results of the in vitro xanthine oxidase inhibitory activity test of PPR extracts showed that the EtOAc fraction exhibited the higher xanthine oxidase inhibitory activity compared to the H_2O fraction with an IC50 value of 38.15 µg/ml and 40.18 µg/ml, respectively. Based on the in vitro xanthine oxidase inhibitory activity test, the ethyl acetate fraction was selected for analysis and chemical structure determination.

Physical constants and spectral data and structure elucidation of isolated compounds of PPR

Compound 1 (PR-1A) was isolated as an amorphous, colorless solid, with a melting point of 269-270 °C. ^{1}H-NMR spectrum of PR-1A was displayed in figure 2a. In the ^{1}H-NMR spectrum of PR-1A, the characteristic signals of a sterol is identified, with 6 methyl groups at δH 0.72 (3H, s, H-18), 1.03 (3H, s, H-19), 0.94 (3H, d, J = 6.5 Hz, H-21), 0.79 (3H, d, J = 7.0 Hz, H-26), 0.87 (3H, d, J = 7.0 Hz, H-27) and 0.80 (3H, t, J = 7.0 Hz, H-29); one oxymethine group at δH 3.60 (1H, m, H-3); a trisubstituted double bond signal at δH 5.24 (1H, brs, H-6) and two protons of an exocyclic double bond at δH 5.17 (1H, dd, J =15; 8.5 Hz, H- 22) and δH 5.04 (1H, dd, J =15.0; 8.5 Hz, H-23). The ^{13}C-NMR appeared to have signals of 35 carbons with characteristic of a sterol skeleton: 6 methyl groups at δC 11.8 (C-18), 19.5 (C-19), 20.9 (C-21), 18.7 (C-26), 20.8 (C-27) và 11.9 (C-29); one oxymethine group at δC 75.7 (C-3); a trisubstituted double bond at δC 140.2 (C-5) 121.9 (C-6); and two methylene groups (CH_2) at δ 33.8 (C-22) and 25.9 (C-23). Present data suggested that PR-1A is β-sitosterol conjugated with one molecule of glucose. Furthermore, an anomeric carbon signal was observed at δC 100.9 (C-1'). The 1H-NMR spectrum also showed three oxymethine proton signals at δH 3.41 (1H, dd, J1=5.0; 8.5 Hz, H-2'), 3.22 (1H, dd, J1=J2= 8.5, H-3'), 3.41 (1H, dd, J1=J2= 8.5 Hz, H-4'). Comparison with published spectral data allowed for the identification of the sugar moiety as β-D-glucopyranose. The spectral data of PR-1A were in full agreement with the evidenced spectral data for β-sitosterol-3-O-β-D-glucopyranoside[25]. Chemical structure of β-sterol-3-O-β-D-glucopyranoside was shown in figure 3a.

Compound 2 (PR-4D1) was isolated as yellow crystalline solids having melting point at 313-314 °C. ^{1}H-NMR spectrum of PR-4D1 was shown in figure 2b. The ^{1}H NMR spectrum has characteristic features of a flavonol skeleton. In five aromatic ring proton signals featured for ABX- type, there are two meta-coupled proton signals at δ_H 6.51 (1H, d, J = 2.0 Hz) and 6.26 (1H, d, J = 2.0 Hz) assigned to H-8 and H-6, respectively, corresponding to the 5,7-dihydroxy structure of ring A; and three aromatic protons at δ_H 7.82 (1H, d, J = 2.0 Hz, H-2′), 6.99 (1H, d, J = 8.5 Hz, H-5′), and 7.69 (1H, dd, J = 8.5, 2.0 Hz, H-6′) belongs to ring B, which suggests substitution at the C-1′, C-3′, and C-4′ positions. The absence of a singlet corresponding to H-3 of the aromatic nucleus further supported substitution at the C-3 position. In the ^{13}C-NMR spectrum, there is a signal at δ_C 136.7, which is feature of a flavonol bearing a OH group at C-3. The ^{13}C-NMR indicated that PR-4D1 comprises 15 carbon atoms. Of these, five CH groups featured aromatic carbons at δ_C 94.2 (C-6), 99.1 (C-8), 115.7 (C-2′), 116.2 (C-5′), and 121.4 (C-6′) ppm and ten quaternary carbons in which the signal at δ_C 176.5 ppm featured for a conjugated carbonyl group (C=O) in a flavonoid skeleton. Four quaternary carbon signals at δ_C 145.8, 146.9, 162.3, and 164.9 ppm were assigned to aromatic carbons bonded to OH groups, corresponding to C-3′, C-2, C-5, and C-7, respectively. These spectroscopic data clearly support that PR-4D1 is a flavonoid. On the basis of the comparison with the published studies allowed to conclude that the identified PR-4D1 as quercetin[26]. Chemical structure of quercetin was shown in figure 3b.

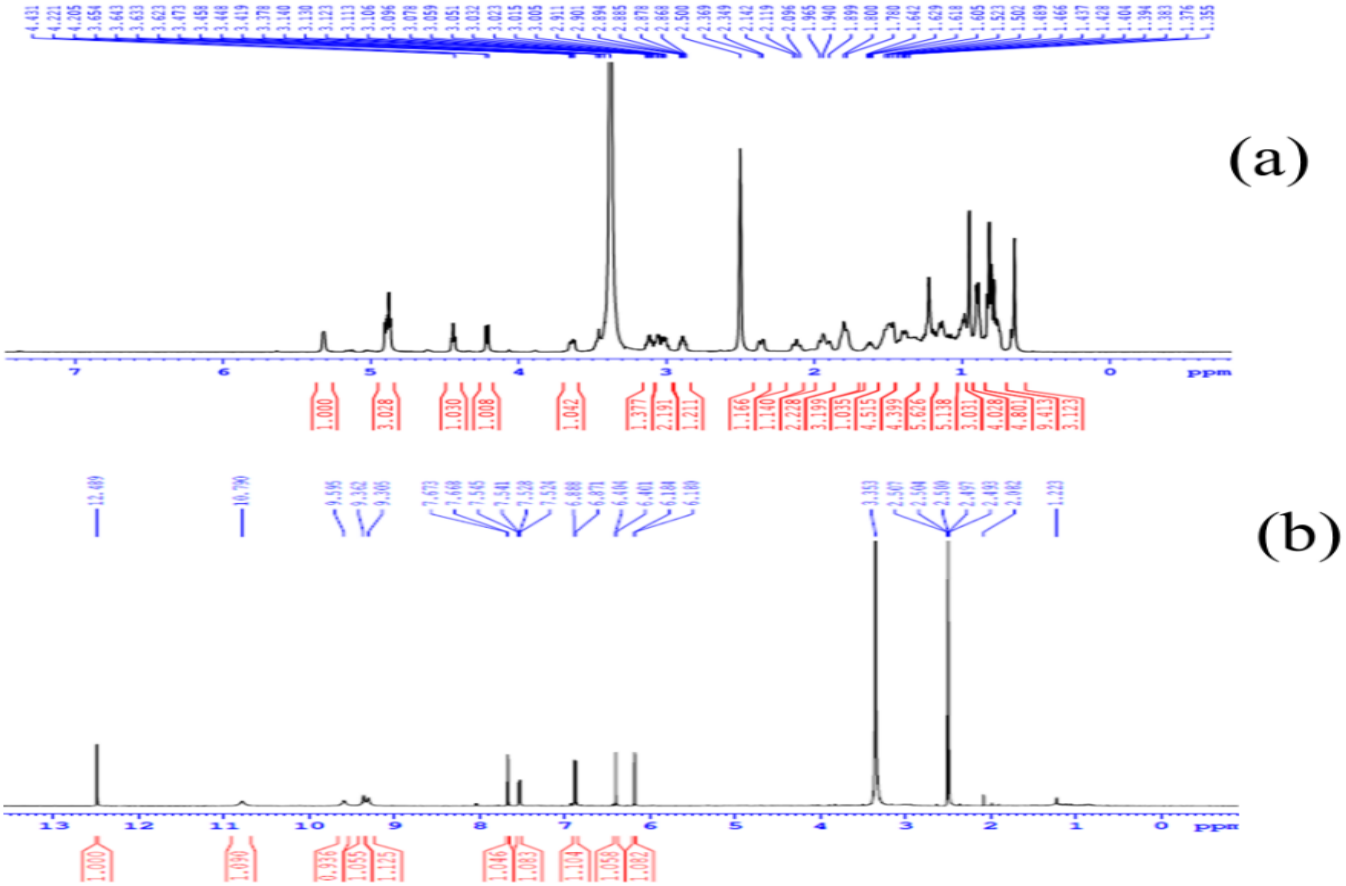

Figure 2. ^{1}H-NMR spectra of β-sitosterol-3-O-β-D-glucopyranosid and quercetin

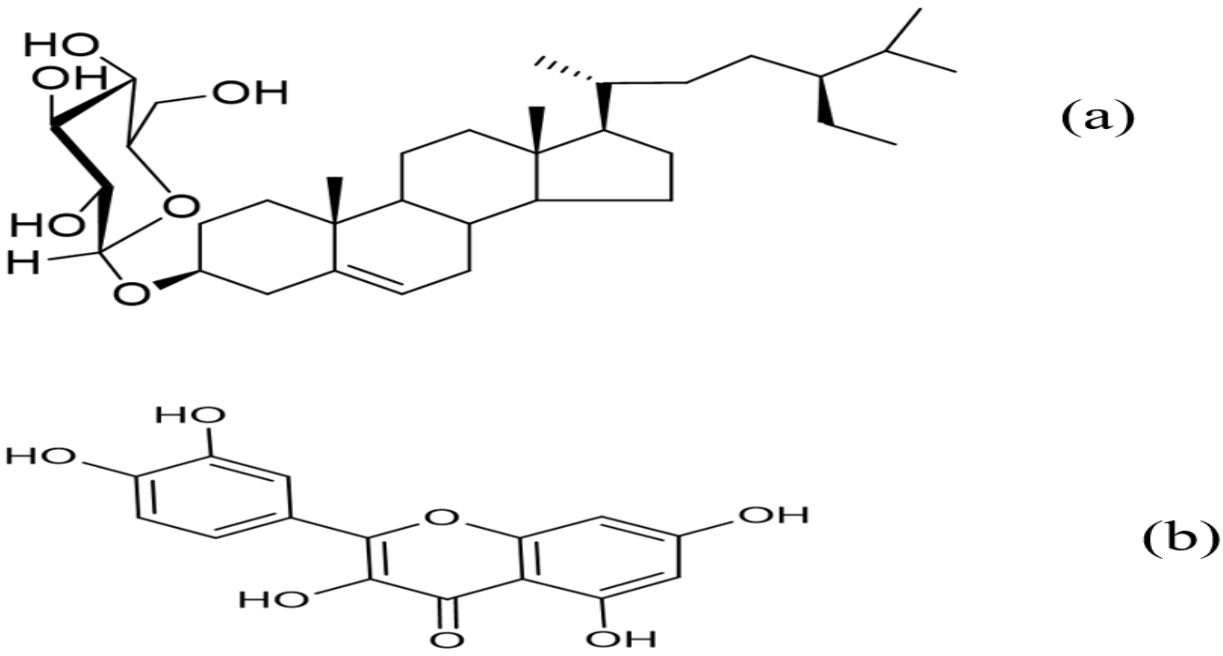

Figure 3. Chemical structure of β-sterol-3-O-β-D-glucopyranoside (a), quercetin (b).

The chlorogenic acid identification of LTF in flowering stages

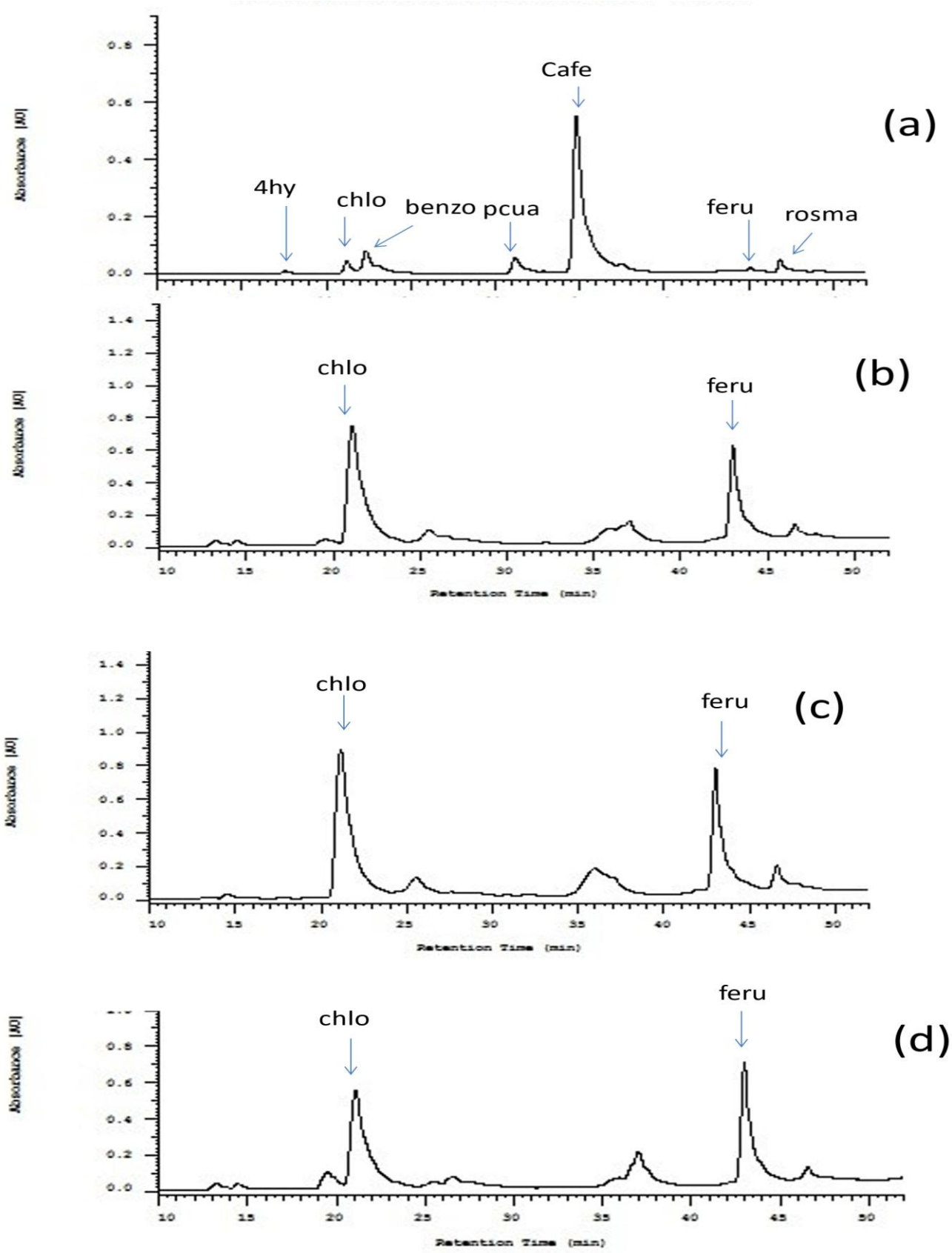

Figure 4. HPLC chromatograms of chlorogenic acid and related phenolic compounds. (a) Standard phenolic compounds; (b) green bud stage; (c) white bud and silver flowering stages; (d) golden flowering stage.

Abbreviations: 4-HB, 4-hydroxybenzoic acid; Chlo, chlorogenic acid; Benzo, benzoic acid; p-Cua, p-coumaric acid; Cafe, caffeic acid; Feru, ferulic acid; Rosma, rosmarinic acid.

The chromatographic profiles of the LTF at different flowering stages are displayed in Figure 4b-d. Chlorogenic acid and ferulic acid were quantified in LTF, with chlorogenic acid being the main active constituent. The content of chlorogenic acid varied across the flowering stages, following the order: white bud and silver flowers (268.43 ± 0.02 mg/g dry extract) > green flowers (202.4 ± 0.01 µg/g dry extract) > yellow flowers (104.3 ± 0.02 µg/g dry extract) (Figure 5), and the content of ferulic acid also is highest in white bud and silver flowering stage (167.23±0.04 µg/g dry extract). More than 140 compounds have been isolated and identified from L. japonica, with major constituents such as chlorogenic acid, isochlorogenic acid, caffeic acid, hexadecanoic acid, and myristic acid[7]. Particularly, ferulic acid was determined for the first time in LTF.

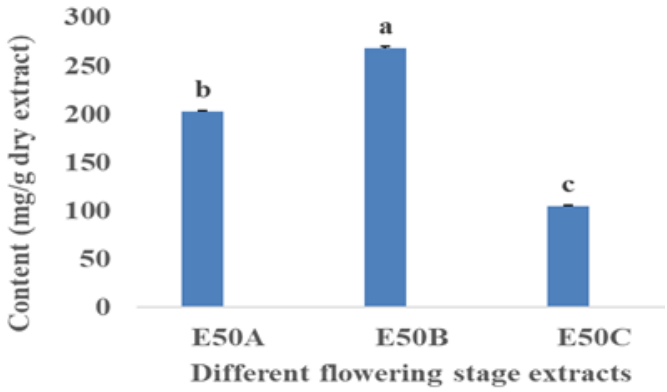

Figure 5. Chlorogenic acid content at different flowering stages of Lonicera japonica Thunb. Values are expressed as mean ± SD (n = 3). Different superscript letters indicate statistically significant differences ($p < 0.05$).

Xanthine oxidase enzyme inhibiting capacity of compounds and extract mixtures

Table 1. Xanthine oxidase enzyme inhibiting capacity of compounds and mixtures

Compound and mixtures	IC_{50} (µg/mL)
Allopurinol*	2.65 ± 0.27^{e}
β-sitosterol-3-O-β-D-glucopyranoside	36.38 ± 0.67^{ab}
Quercetin	28.07 ± 1.23^{c}
Chlorogenic acid	22.0 ± 0.22^{d}
Ferulic acid	3.60 ± 0.32^{e}
PPR-MeOH	42.12 ± 0.34^{a}
E50B	33.89 ± 1.36^{ab}

Note. Values are expressed as mean ± SD (n = 3). Different superscript letters indicate statistically significant differences ($p < 0.05$). *Positive control substance.

The XO inhibiting capacity in vitro of compounds and extracts was evaluated by the IC_{50} values (Table 1). The results were found that XO inhibitory effect reduced in the following order: ferulic acid > chlorogenic acid > quercetin> E50B> β-sitosterol-3-O-β-D-glucopyranoside > PPR-MeOH. As of now, β-sitosterol-3-O-β-D-glucopyranoside, PPR-MeOH and E50B were firstly tested XO inhibition capacity.

Extract mixture induced growth inhibition in A549 cells

PPR-MeOH, E50B, and their combined formulation (PPR-MeOH and E50B, 1:1, v/v) significantly induced cytotoxic effects against A549 cells. The growth-inhibitory potency against A549 cells reduced in the following order: fluorouracil > PPR-MeOH: E50B mixture (1:1) > PPR-MeOH > E50B (Table 2). Especially, the combined extracts presented the stronger antiproliferative effect compared to either extract alone, which recommended a potential synergistic or additive interaction between PPR-MeOH and E50B. In former works, Lonicera japonica Thunb had been evidenced significantly enhancing bioactivity when combined with other medicinal herbs such as co-administration with Oroxylum indicum[27], in combination with Astragalus membranaceus[28], and Magnolia obovata[29]. These synergistic effects should furnish insightful understanding support for the rational development of multi-herb formulations for natural remedy applications. The previously in vitro researches strongly evidenced that both crude extracts and isolated compounds from P. polyphylla var. chinensis displayed tumor growth–suppressive activity of cell lines such as MCF-7 breast cancer cell[30,31,32], HepG2[31], MDA-MB-231[32], HL-60, A549/ATCC, SW-620, melanoma M14, and renal cancer 786-0 cell lines[33]; However, further mechanistic researches and clinical validation are essential to fully establish its therapeutic potential.

Table 2. Cytotoxicity of extracts against A549 cells after 24 hr

Compounds	IC_{50} (µg/mL)
Flourouracil*	2.29 ± 0.22^{d}
PPR-MeOH	6.32 ± 0.17^{b}
E50B	10.30 ± 0.23^{a}
PPR-MeOH and E50B miture (at ratio 1:1)	4.11 ± 0.16^{c}

Note. Values are expressed as mean ± SD (n = 3). Different superscript letters indicate statistically significant differences ($p < 0.05$). *Positive control substance.

PPR-MeOH and E50B mixture altered the expression of apoptosis-related proteins A549 cells

The expression of apoptosis-related proteins in A549 cells was detected by Western blotting (Figure 6). The results highlighted that treatment with the PPR-MeOH and E50B mixture markedly activated caspase-3 and caspase-9 and enhanced cleavage of p53. The tumor suppressor gene p53 is a vital regulator of apoptosis and a crucial barrier against tumor development[34,35].

Activated caspase-9 and caspase-3 are important executors of the intrinsic apoptotic pathway in cancer cells. Caspase-9 is activated following mitochondrial Cytochrome c release, subsequently triggering caspase-3 activation, which mediates proteolytic cleavage of critical cellular substrates and results in apoptotic cell death[36,37]. These discoveries strongly emphasize that the combination of LTF and PPR induces apoptosis in A549 cells, which provides robust evidence for the lung anticancer potential of the LTF–PPR mixture and recommends a scientific rationale for the traditional use of them to treat lung cancer in Vietnamese folk medicine.

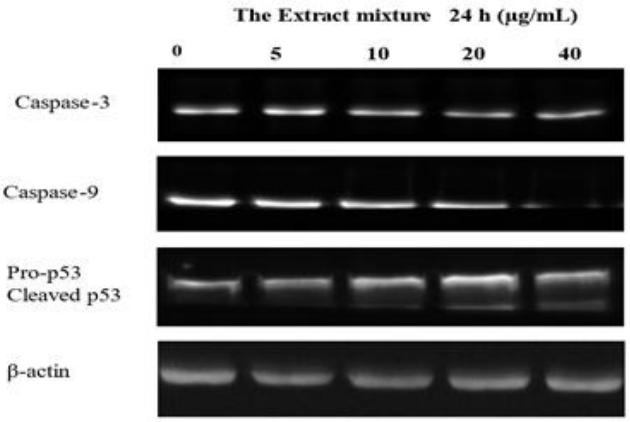

Figure 6. PPR-MeOH and the E50B mixture significantly modulated the expression of apoptosis-related proteins, including caspase-3, caspase-9, and p53, in A549 cells after 24 h of treatment

CONCLUSION

In summary, ferulic acid is the first to identify from the LTF. The XO inhibitory capacity of the isolated compounds and extracts, and A549 cell growth-inhibitory effects of the LTF–PPR mixture are also firstly investigated. PPR-MeOH and E50B miture (at ratio 1:1) presented the stronger antiproliferative effect compared to either extract alone. Authors call for research strategy on the synergistic effects by demonstrating molecular mechanisms models and perform rigorous quantitative evaluation of the synergistic effects of the herbal pair.

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Conflict of interest: There are no competing financial interests.

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