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Separation of Non-alkaloid Toxin Lignans and New Flavonoid from Himalayan Mayapple (*Podophyllum Hexandrum* Royle) by High-Speed Counter-Current Chromatography and Their Anti-inflammatory Activity Evaluation

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Abstract

Podophyllum hexandrum Royle (Berberidaceae) is reported from the Himalayan region and China. It is also known as the Himalayan Mayapple and is reported for the treatment of constipation, fever, jaundice, liver disorders, etc. Herein, the isolation of chemical constituents using high-speed counter-current chromatography (HSCCC) from the % Ethanol (EtOH) extract of the rhizomes of Himalayan Mayapple is reported. As a result, kaempferol 3-glucoside (1), quercetin-3-O-*β*-D-glucopyranoside (2), quercetin 3-O-*β*-D-glucopyranosyl-(1→6)-3-*O*-ethyl-*β*-D-glucopyranoside (3), kaempferol 3-O-*β*-D-glucopyranoside (4), *α*-peltatin(5), podophyllotoxin (6), 4'-demethylpodophyllotoxin (7), 4',5'-didemethylpodophyllotoxin (8), and kaempferol (9)were separated. Compounds 6-9 were separated by the normal HSCCC while 1-5 were obtained by the offline-recycling HSCCC using n-hexane, Ehyl Acetate, Methanol and Water % (HEMW at 1:9:4:6, *v/v*) solvent system. The pure components were tested in lipopolysaccharides-induced mice macrophage cells. Compounds 6 and 7 showed significant inhibition. The nitric oxide production was inhibited by compounds 6 and 7, effectively, with IC_{50} values of 1.328 x 10^{-6} and 2.851 x 10^{-6} M, respectively. In this assay, kaempferol (9), a positive inhibitor expressively inhibited lipopolysaccharides-induced nitric oxide production.

Keywords: Flavonoids, HSCCC, *Podophyllum hexandrum* Royle, Podophyllotoxins, Mice macrophage cells

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Introduction

The Himalayan Mayapple, *Podophyllum hexandrum* Royle (Berberidaceae), is a perennial medicinally valued plant endemic to the Himalayan region [1] and also found in China [2]. It is used for the treatment of constipation, fever, etc. [1]. The Himalayan

Mayapple is reported for anti-obesity [3], antioxidant [4], antimicrobial, anticancer, anti-inflammatory, antifungal, radio-protective [1], and radioprotectant [5] properties. The main chemical constituent, podophyllotoxin reported from the Himalayan Mayapple has played a vital role in drug discovery for cancer chemotherapy [2] and it has shown activities like antifungal, antiviral, etc. [6]. The plant species contains non-alkaloid toxin lignans and flavonoids [7]. A significant class of nutraceuticals, lignans, have shown their efficacy in chronic disorders [8]. Podophyllotoxin, a naturally sustainable anticancer ligan, contributes to the industrial value and its derivatives have been reported to show promising acaricidal and insecticidal properties [9].

Traditional methods like Sephadex and ODS [10, 11] silica gel [11], HPTLC [12], micellar electrokinetic chromatography [13], reversed-phase HPLC [14], etc. have been reported for the isolation of compounds from *P. hexandrum*. In addition, HSCCC has also been reported for lignans [15, 16] and flavonoids [10] separation from *P. hexandrum*. However, the previously reported methods based on HSCCC are supported by HPLC-MS and medium-pressure liquid chromatography [15], Sephadex [10], and silica gel [17]. The present study is solely based on HSCCC, and interestingly, the lignans and flavonoids are separated in a single experiment.

Owing to the therapeutic value of the non-alkaloid toxin lignans viz. α-peltatin, podophyllotoxin, 4'-demethylpodophyllotoxin, 4',5'-didemethylpodophyllotoxin etc., and flavonoids likely kaempferol and quercetin derivatives, the present study explored the constituents of *P. hexandrum*. Herein, the HSCCC technique is reported to separate the secondary metabolites from the ethanol extract of *P. hexandrum*. As a result, nine compounds (Fig. 1) were separated using a normal and offline-recycling mode HSCCC.

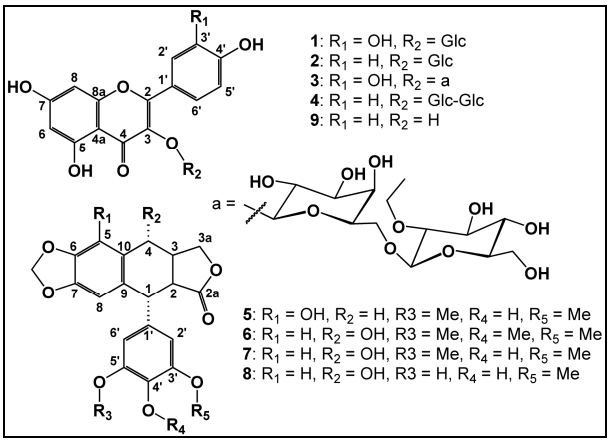

Figure 1. **Phytoconstituents from *P. hexandrum***

The compounds were characterized by spectral techniques. Further, *in vitro* anti-inflammatory activities were also conducted for the pure components.

Materials and Methods

The analytical grade *n*-hexane, Ethylacetate (EtOAc) and Methanol (MeOH) (Sinopharm Chemical Reagent Co., Ltd., China), Acetonitrile (CAN) (Fisher Company, USA) were used. Milli-Q system (Millipore, USA) was used to get H_2O.

HSCCC (EMC-300) instrument (Emilion Technology, China) with 300 mL capacity in three columns (2.6 mm i.d.) and a loop (20 mL) was further connected with pump (TBP-5002), water bath (DC-0506), UV monitor (8823A), and a recorder (Model 3057) from Tauto Biotechnique (Shanghai, China), Emilion Technology (Beijing, China), and Instrument Factory (Sichuan, China), respectively. HPLC system (1260) from Agilent Technologies (USA) was used for sample analysis.

The rhizomes were purchased from the Gilgit market and identified by Professor

Sher Wali Khan. The specimen (KIU-21-Ch88) was kept in the Department of Biological Sciences, Karakoram International University.

P. hexandrum (1.5 kg, rhizome part) was extracted three times with 2 × 5 L (90% ethanol) for 48 h. The crude (80 g) was obtained at 40 °C in vacuo.

Solvent System and Partition Coefficients

A combination of four solvents i.e. *n*-hexane / EtOAc / MeOH / H_2O (HEMWat) making two phases was prepared in a tube. Each phase, in equal amounts, was taken out in another tube to dissolve 2 mg of the crude. After complete dissolution, the tube was kept stand-by until the clear separation of phases. Then 1 mL of each phase containing the dissolved sample was taken and dried in tubes. Then 1 mL of methanol was used to dissolve each dried sample and analyzed (HPLC). The HEMWat (1:9:4:6, *v*/*v*) mixture was employed for the experiments. The solvent mixture was prepared in a separating funnel. After some time, the upper (stationary), and lower (mobile) parts were separated. To run the experiment, the crude (300 mg) was dissolved in a 20 mL isometric mixture, and the sample was found completely soluble in the mixture. The two-phase HEMWat (1:9:4:6, *v*/*v*) was also applied for the offline-recycling HSCCC of peak I.

HSCCC Procedure, HPLC Analysis and Characterization

Primarily, the Counten Current Chromatography (CCC) column was filled with the stationary phase. The instrument was switched on (800 rpm, clockwise), and the mobile phase was pumped (3.0 mL/min). The instrument was set for the temperature of the water bath (24 °C) and UV (254 nm). The forward-inlet (FWD-IN) elution mode was applied. At equilibrium, the stationary phase retention (calculated) was found 88.57%. The sample already dissolved in the optimized mixture was injected at hydrodynamic equilibrium. The elutions (10 mL each) were collected by automatic sampler.

The mobile phase (A: H_2O, B: ACN) was applied at 254 nm with 1 mL/min (flow rate): 0–1 min, isocratic 70% A; 1–6 min, linear 70%–50% A; 6–13 min, linear 50%–10% A; 13–14 min, linear 10%–50% A; 14–15 min, linear 50%–70% A; 15–18 min, isocratic 30% B. For mass spectra, Agilent 6520 Q-TOF (Agilent, Santa Clara, CA, USA) was used. For NMR Bruker AV-400 spectrometer (Bruker BioSpin, Germany) was used.

NO Inhibition Rate in Macrophage Cells

Griess method nitric oxide (NO) detection kit from Shanghai Biyuntian Biotechnology Co. Ltd. (S0021) was used. Then 50,000 mice macrophage cells (RAW264.7) were seeded on a culture plate (96-well) in 100 μL medium. Then 200 ng/mL lipopolysaccharide (LPS) was added after 24 h. Further, it was incubated for 24 h. The sample and the standard (60 μL) were shifted to a new culture plate. To each well, Griess Reagent I and II (60 μL) were added. The absorbance (540 nm) was checked by a microplate reader. The standard curve method was applied to calculate NO concentration, and the NO production inhibition rate was determined.

Statistical Analysis

The obtained results were revealed as means ±SD. The significant values (p-values < 0.05) were determined by one-way ANOVA using GraphPad Prism.

Results and Discussion

Solvent Mixture and Separation

HSCCC separation requires a particular biphasic solvent mixture [18]. The HEMWat solvent mixtures were applied. Principally, the two-phase HEMWat solvent system contains *n*-hexane/EtOAc in the stationary phase and methanol/water in the lower phase. The solvent ratios for the stationary phase were kept constant while the lower phase solvent ratios were varied, and vice versa. Thus, some HEMWat solvent systems were tested. As shown in Table 1, in HEMWat (1:1:1:1, *v*/*v*) solvent system, 1-3, and 9 showed partition coefficients (<0.5) and 5 and 4 showed K_D of 0.83 and 1.72, respectively. While 6-8 showed K_D greater than 5. Hence this system was applicable for the separation of 4 and 5. When the methanol in the lower phase methanol:water ratio was decreased to 3:7, the K_D of most of the compounds was found greater than 5. Later, the ethyl acetate in the stationary phase was increased to 4:6, and then 1:9. The decrease in the ethyl acetate in the upper part showed the K_D of most of the compounds in the range greater than 5. Thus, finally keeping HEMWat (1:9:4:6, *v*/*v*) solvent system, the K_D for 6-9 were found 0.98, 1.32, 2.02, and 3.19, respectively. While compounds 1 and 2 exhibited K_D of 0.21 and 0.28, respectively. The K_D of 3-5 was found lesser than 0.5. Therefore, HEMWat (1:9:4:6, *v*/*v*) solvent mixture yielded 6-9 in the normal HSCCC experiment. Compounds 1-5 were further separated in pure form using the same solvent system, HEMWat (1:9:4:6, *v*/*v*), by the offline-recycling mode.

Table 1. **The partition coefficients of compounds in HEMWat (1:9:4:6, v/v) two-phase solvent system.**

HEMWat	K_D-values								
	1	2	3	4	5	6	7	8	9
5:5:5:5, *v*/*v*	<0.1	0.23	0.25	1.72	0.83	8.05	>10	>10	<0.1
5:5:3:7, *v*/*v*	2.19	5.97	6.10	9.01	5.65	6.45	9.35	3.38	>10
8:2:3:7, *v*/*v*	1.25	5.19	4.58	4.02	7.26	>10	0.57	>10	>10
4:6:3:7, *v*/*v*	2.18	5.17	4.88	4.96	2.00	5.45	7.46	3.11	>10
1:9:4:6, *v*/*v*	0.21	0.28	<0.1	<0.1	<0.1	0.98	1.32	2.02	3.19

The crude (300 mg) of *P. hexandrum* was dissolved and loaded in elution mode using HEMWat (1:9:4:6, *v*/*v*) solvent system. The HSCCC chromatogram (Fig. 2) showed 5 peaks (I-V) with two smaller peaks in between peaks I and II that did not contain any compounds. Peak I contained the mixture, and offline-recycling mode was applied to separate the mixture into pure compounds.

As shown in Fig. 2, peak I consisted of a mixture of five (1-5) and it was stored & recycled using HEMWat (1:9:4:6, *v*/*v*) solvent system with a lesser flow rate. The peaks II-V were found to contain pure compounds 6-9, respectively, and were obtained in a single run. Furthermore, the offline-stored peak I was recycled as shown in Fig. 3.

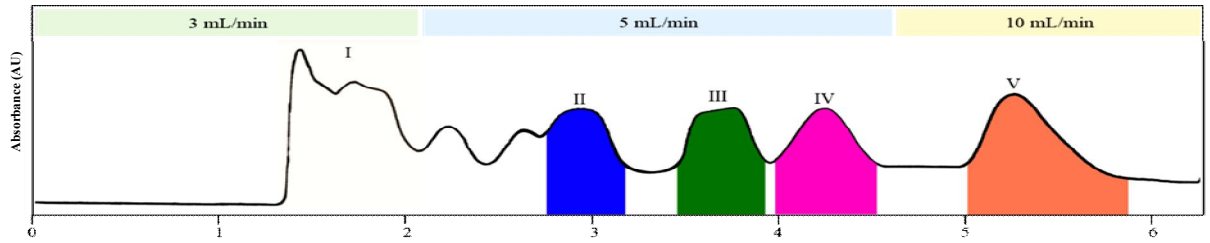

Figure 2. **HSCCC chromatogram for separation of compounds from *P. hexandrum*; Solvent system: HEMWat (1:9:4:6, v/v); Flow-rate: 3.0 mL/min for 125 min, 5.0 mL/min for 150 min, 10.0 mL/min for 110 min; Detection wavelength: 254 nm; Rotation speed: 800 rpm; Separation mode: FWD-IN; Sample loading: 300 mg**

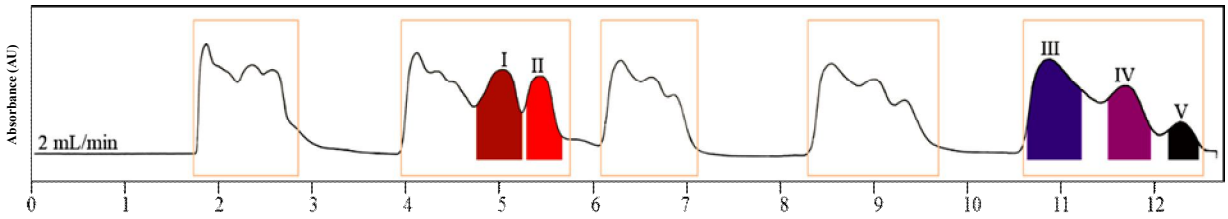

Figure 3. **Offline-recycling HSCCC chromatogram for separation of compounds (1-5); Solvent system: HEMWat (1:9:4:6, v/v); Flow-rate: 2.0 mL/min; Detection wavelength: 254 nm; Rotation speed: 800 rpm; Separation mode: FWD-IN; 150 mg**

As a result, compounds 3 (peak I in Fig. 3) and 4 (peak II in Fig. 3) were obtained pure in the second cycle. While compounds 1 (peak III in Fig. 3), 2 (peak IV in Fig. 3) and 5 (peak V in Fig. 3) were obtained in the fifth cycle. HPLC analysis showed purity of components greater than 96% as shown in Fig. 4.

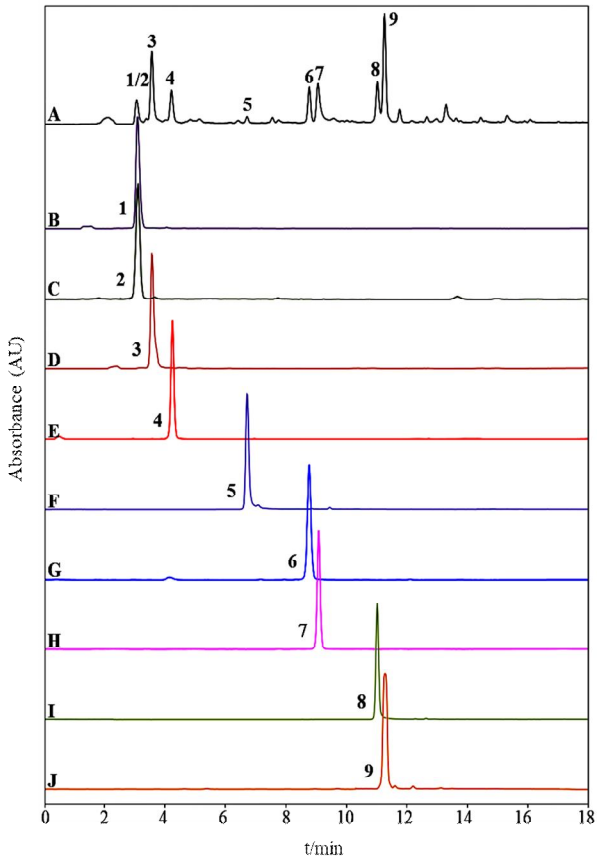

Figure 4. **HPLC chromatograms of the crude sample and the isolated pure compounds. Experimental conditions: Waters Symmetry C_{18} column (5 µm, 4.6 mm× 250 mm, i.d.,); Mobile phase: acetonitrile and water; Flow-rate: 1.0 mL/min; Detection wavelength: 254 nm; Injection volume: 10 µL. A: crude (MeOH) extract; B-J: the isolated pure compounds (1-9)**

Structure Identification

Kaempferol 3-glucoside (1) [19, 20]. (Peak III in Fig.3; Fig. 4B): ESI-MS (negative ion mode) m/z 447.0957.. Compound was characterized as kaempferol 3-glucoside ($C_{21}H_{20}O_{11}$).

Quercetin-3-*O*-*β*-D-glucopyranoside (2) [21, 22]. (Peak IV in Fig.3; Fig. 4C): ESI-MS (negative ion mode) m/z 463.1049. The isolated compound was confirmed as quercetin-3-O-β-D-glucopyranoside with MF $C_{21}H_{20}O_{12}$.

Quercetin 3-O-*β*-D-glucopyranosyl-(1→6)-3-O-ethyl-β-D-glucopyranoside (3). (Peak I in Fig.3; Fig. 4D): ESI-MS (negative ion mode) m/z 653.2726. The molecular formula $C_{29}H_{34}O_{17}$ of compound 3 was confirmed via ESI-MS (negative ion mode) that gave $[M-H]^+$ ion at m/z 653.2726 (calculated for $C_{29}H_{33}O_{17}^+$, m/z 653.171226). The chemical shift values in the NMR spectra were found mostly similar to that of the reported compound namely quercetin-3-O-β-D-glucopyranosyl-(1-6)-β-D-glucopyranoside [23]. However, two additional carbon atoms, resonating as methyl at δ_C 15.4 ppm and methylene at δ_C 66.1 ppm were found connected to the glucopyranoside moiety. The sugar proton resonating at δ_H 4.26 (1H, d, J = 7.8 Hz, H-1''') demonstrated a strong correlation to the methylene carbon at δ_C 66.1 ppm as shown in Fig. 5. Thus, the COSY and HMBC spectral analysis confirmed the ethyl group (as ethoxy) attachment to the sugar

moiety at C-1'''. To the best of our knowledge, compound 3 was found as a new compound.

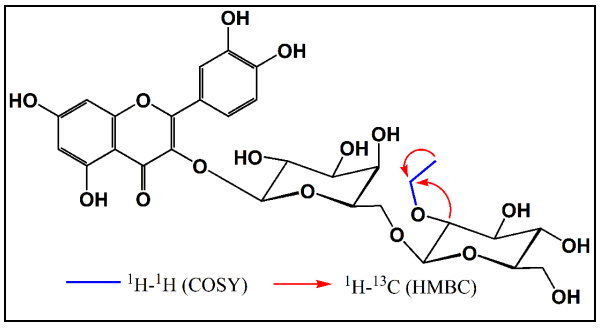

Figure 5. **2D-NMR correlations of compound 3**

Kaempferol 3-O-β-D-glucopyranoside (4) [24]. (Peak II in Fig. 3; Fig. 4E): ESI-MS (positive ion mode) m/z 611.1410. The isolated compound was further confirmed as kaempferol-3-sophoroside ($C_{27}H_{30}O_{16}$).

α-Peltatin (5) [25, 26]. (Peak V in Fig.3; Fig. 4F): ESI-MS (positive ion mode) m/z 401.1227. The structure was deduced to be α-peltatin with MF $C_{21}H_{20}O_8$.

Podophyllotoxin (6) [26]. (Peak II in Fig. 2; Fig. 4G): ESI-MS (positive ion mode) m/z 415.0851. The structure was elucidated as podophyllotoxin ($C_{22}H_{20}O_8$).

4'-Demethylpodophyllotoxin (7) [26]. (Peak IV in Fig. 2; Fig. 4H): ESI-MS (negative ion mode) m/z 399.1099. The structure was determined as 4'-demethylpodophyllotoxin ($C_{21}H_{20}O_8$).

4',5'-Didemethylpodophyllotoxin (8) [27]. (Peak III in Fig. 2; Fig. 4I): ESI-MS (negative ion mode) m/z 385.1915. The structure was confirmed as 4',5'-didemethylpodophyllotoxin ($C_{20}H_{18}O_8$).

Kaempferol (9) [28]. (Peak V in Fig. 2; Fig. 4J): ESI-MS (negative ion mode) m/z 285.0421. The compound was found as kaempferol ($C_{15}H_{10}O_6$).

Anti-inflammatory Activity

The inhibition of NO production was tested by the effects of the pure components in cells, in the presence of lipopolysaccharides (LPS). The pure compounds i.e. kaempfero l 3-glucoside (1), quercetin-3-O-β-D-glucopyranoside (2), quercetin 3-O-β-D-glucopyranosyl-(1→6)-3-O-ethyl-β-D-glucop-yranoside (3), kaempferol 3-O-β-D-glucopyr-anoside (4), α-peltatin (5), podophyllotoxin (6), 4'-demethyl-podophyllotoxin (7), 4',5'-didemethylpodoph-yllotoxin (8), and kaempferol (9) were studied at the test concentrations 100 µM. All pure components were evaluated in mice macrophage cells except DMSO. The compound kaempferol (9), isolated pure was kept as a positive inhibitor.

Compounds 1-9 significantly inhibited NO production effects in cells (Fig. 6). Among them, compounds 6 and 7 showed the most significant inhibition at doses 100 µM, in the presence of LPS. These compounds were further tested to find their IC_{50} values of NO inhibition. They were tested at doses 50, 10, 2, 1, 0.5, 0.1, 0.05 µM. It was found that 6 and 7 effectively inhibited NO production, with IC_{50} values of 1.328×10^{-6} and 2.851×10^{-6} M, respectively.

A previously reported study describes that the compounds of P. hexandrum play a vital role in radiation-induced inflammation [5]. Furthermore, the alcoholic fraction of P. hexandrum has also shown anti-inflammatory activity by effectively regulating inflammation and cell death pathways [29]. These findings strongly support the anti-inflammatory activity of isolated compounds of P. hexandrum by NO inhibition in the current study.

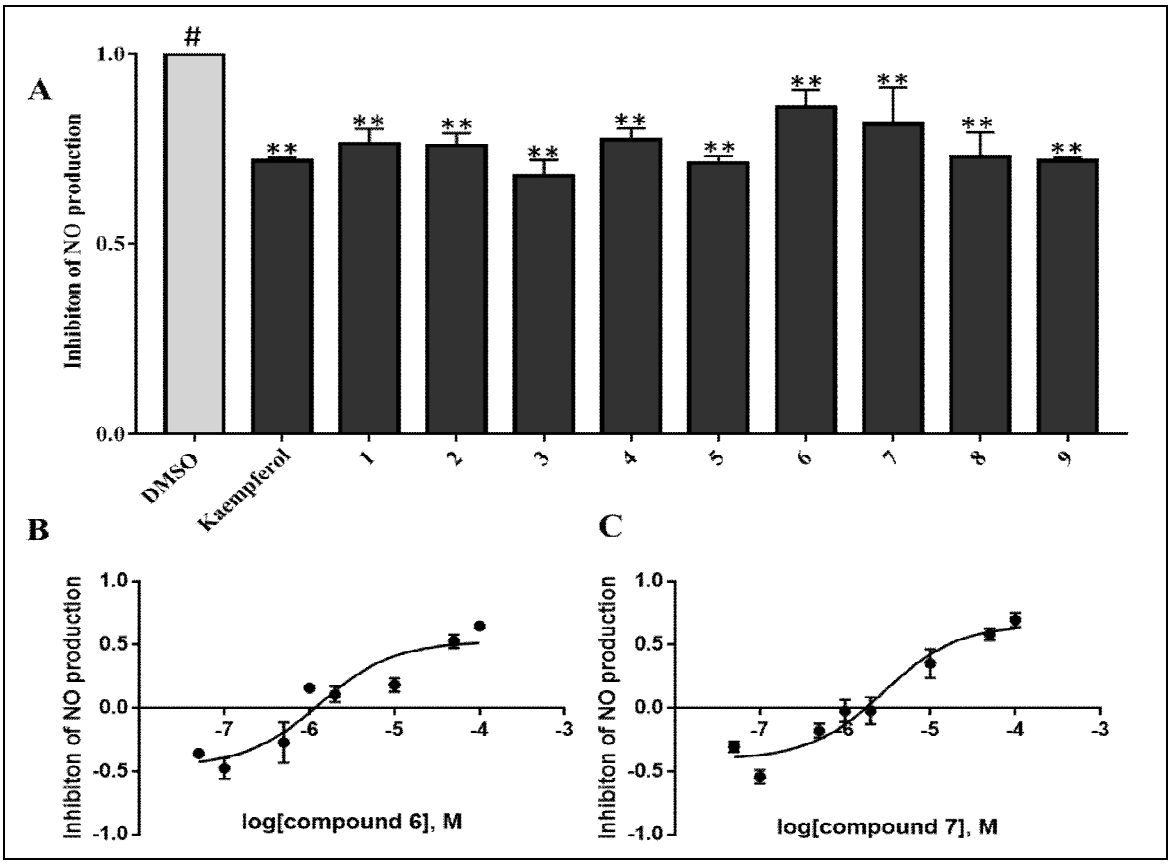

Figure 6. **Effects of components from *P. hexandrum* on LPS-induced oxidative stress in mice macrophage cells. A: Inhibition of NO production by compounds 1-9 at doses 100 μM in the presence of LPS. Compound 9 was used as positive inhibitor. B: Inhibition of NO production by compound 6 at 50, 10, 2, 1, 0.5, 0.1, 0.05 μM in the presence of LPS. C: Inhibition of nitric oxide production by compound 7 at 50, 10, 2, 1, 0.5, 0.1, 0.05 μM in the presence of LPS**

Conclusion

Lignans and flavonoids were successfully separated by HSCCC technique in elution mode followed by offline-recycling, using a solvent system composed of *n*-hexane:ethyl acetate:methanol:water (1:9:4:6, *v*/*v*). The results revealed the presence of flavonoids like kaempferol 3-glucoside, quercetin-3-O-*β*-D-glucopyranoside, quercetin 3-O-*β*-D-glucopyranosyl-(1→6)-3-*O*-ethyl-*β*-D-glucopyranoside, kaempferol 3-O-*β*-D-glucopyranoside, and kaempferol, and lignans including *α*-peltatin, podophyllotoxin, 4'-demethylpodophyllotoxin, and 4',5'-didemethylpodophyllotoxin. The anti-inflammatory evaluation revealed that two lignans viz. podophyllotoxin, and 4'-demethylpodophyllotoxin were found the most active, compared to a flavonoid i.e., kaempferol, a positive inhibitor. The present study also revealed the separation of a new flavonoid for the first time. Compared to the previous reports, lignans and flavonoids were successfully separated by using the optimized HSCCC solvent system, for the first time. The optimized solvent system might also be employed to separate chemical constituents from other herbal sources.

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Conflict of Interest

Authors declare that there is no conflict of interest.

Data Availibilty Statement

The related NMR raw data can be obtained from PJEAC or Corresponding Author on request.

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