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ORIGINAL PAPER

Preparative separation of polymethoxylated flavones from Ponkan (*Citrus reticulata* Blanco cv. Ponkan) peel by high-speed countercurrent chromatography and their antifungal activities against *Aspergillus niger*

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Abstract Three polymethoxylated flavones (PMFs) including nobiletin (NOB), tangeretin (TAN) and 5-demethylnobiletin (5-DN) were separated from Ponkan (*Citrus reticulata* Blanco cv. Ponkan) peel by high-speed countercurrent chromatography (HSCCC) with a two-phase solvent system composed of *n*-hexane–ethyl acetate–methanol–water (1:0.8:1:1, v:v:v:v). A total of 6.4 mg of NOB, 20.2 mg of TAN and 2.8 mg of 5-DN with the purity of 97.7, 99.05 and 95.9 %, respectively, were obtained in one-step separation from 70 mg of crude extracts. Their chemical structures were identified by HPLC/ESI–MS and ^{1}H and ^{13}C-NMR. The antifungal activities of the three compounds against *Aspergillus niger* were evaluated by using a microbroth dilution assay. 5-DN displayed the best inhibitory effect, while NOB showed the lowest potency, with respective minimal inhibitory concentration (MIC) values of 0.1 and 0.8 mg/mL, which indicated that the inhibition against *A. niger* could be affected by the structural difference in PMFs.

Keywords Ponkan · Polymethoxylated flavones · High-speed countercurrent chromatography · Antifungal activity · Structure–activity relationship

Abbreviations

PMFs	Polymethoxylated flavones
HSCCC	High-speed countercurrent chromatography
NOB	Nobiletin
TAN	Tangeretin
5-DN	5-Demethylnobiletin
HPLC	High-performance liquid chromatograph
PDA	Photodiode array detector
ESI-MS	Electrospray ionization–mass spectrometry
NMR	Nuclear magnetic resonance
MIC	Minimal inhibitory concentration

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Introduction

Polymethoxylated flavones are a general term for flavones bearing four or more methoxy groups on their basic benzo-γ-pyrone (15-carbon, C6–C3–C6) skeleton with a carbonyl group at the C4 position. They are almost exclusively found in the citrus genus, particularly in the peels of mandarins and sweet oranges [1]. Ponkan is produced in Asia, known throughout the world as the thick-skinned mandarin orange. Ponkan peel is a kind of potential rich source of PMFs.

Polymethoxylated flavones have been of particular interest due to their broad spectrum of biological activities, including anti-inflammatory [2, 3], antioxidant [4], anti-allergic [5], antiproliferative [6] activities and effects on mammalian metabolism [1]. Recent studies report that PMFs are also potent inhibitors of microorganism growth, having antibacterial [7], antifungal [8] and antiviral [9] activities, and these activities are directly related to their chemical structure, depending on the number and positions of hydroxyl groups and methoxyl groups [10–12]. Due to the trend of consumer preferences toward more natural and healthier products, scientific research has begun to focus on

screening natural antimicrobial compounds as biopreservatives. Previous research in the literature mainly focused on the antibacterial or antiviral activities; however, information is lacking regarding PMFs' antifungal capacity and structure–activity relationship.

The purification of PMFs is critical for further studies of their biological activities and structure–activity relationships. HSCCC is a kind of liquid–liquid partition chromatography. The liquid stationary phase is immobilized in the column by centrifugal force. When the mobile phase is pumped through the column, sample components are partitioned between the two phases and they are separated on the basis of difference in partition coefficients. HSCCC eliminates irreversible adsorption of the sample onto the solid support used in a conventional chromatographic column and has a large-scale injection. This method has been widely used in preparative isolation of pure compounds from natural materials [13–15].

In the present study, the isolation and purification of PMFs in Ponkan peels by HSCCC, and identification of structures by HPLC/ESI–MS and ^{1}H and ^{13}C-NMR were performed. Three kinds of PMFs were obtained, and their antifungal activities against *Aspergillus niger*, a common fungal contaminant of food, were evaluated by using a microbroth dilution assay to compare the influence of PMFs' structural difference on the antifungal activity. The results of this study provide new experimental data for the future exploitation and utilization of PMFs as natural biopreservatives and will be helpful in understanding the mechanisms of PMFs in inhibition against fungi.

Materials and methods

Plant materials

Ponkan peels at mature stage were provided by Yichang Citrus Station, Hubei, China.

Chemicals

All chemicals in the investigation are of analytical grade, which were purchased from Sinopharm Chemical Reagent Co. (Shanghai, China), except that methanol is of chromatograph grade (Fisher, Fairlawn, NJ, USA) used in HPLC–PDA and HPLC/ESI–MS.

Extraction of crude PMFs

The citrus peels were oven-dried at 40 °C until constant weight was reached and milled into a powder (particle size, 0.425 mm). Approximately 100 g of powder was extracted using 95 % ethanol (1,000 mL) at 45 °C for 12 h. The suspension was concentrated and treated with petroleum ether (200 mL × 3) in a separatory funnel. The combined petroleum ether extracts were washed with 0.4 % sodium hydroxide solution until the aqueous fraction was colorless. The petroleum ether layer was collected, concentrated and freeze-dried to yield crude PMFs, which was subjected to subsequent HSCCC separation.

HSCCC separation procedure

The HSCCC system used for PMFs' purification consisted of the following components: a model TBE-300B HSCCC (Shanghai Tauto Biotech Co., Ltd., Shanghai, China) with three polytetrafluoroethylene (PTFE) preparative coils (i.d. of the tubing, 1.5 mm; total volume, 280 mL), a 20-mL sample loop, a model TBP-5002 constant-flow pump, a model TBD-2000 UV detector and a model HW chromatography workstation (Shanghai Kingdom Biochemical Instrument Co., Ltd., Shanghai, China).

The purification of crude PMFs was performed by HSCCC as previously reported by Wang et al. [16]. For the HSCCC separation of the crude PMFs, the solvent system was composed of *n*-hexane–ethyl acetate–methanol–water (1:0.8:1:1, v:v:v:v), with the organic upper phase used as the stationary phase. The sample solution was prepared by dissolving 70 mg of crude PMFs in 7 mL of the mobile phase. For each run, the coil column was initially filled with the stationary phase. Then, the apparatus was rotated at 800 rpm, while the mobile phase was pumped into the column at a flow rate of 1.5 mL/min. After the mobile phase front emerged and hydrodynamic equilibrium was established in the column, the sample solution was injected. The separation temperature was controlled at 25 °C. The effluent was continuously monitored by a UV detector at 340 nm. Each peak fraction was manually collected according to the chromatogram and then evaporated and freeze-dried.

HPLC–PDA analysis

The HPLC system (Waters, USA) consisted of a 2695 separations module, a 2998 photodiode array detector (PDA) and an empowered HPLC workstation. The crude PMFs and each peak fraction from HSCCC were analyzed by HPLC with a Sepax Amethyst C18-H column (250 × 4.6 mm, i.d. 5 μm) at a column temperature of 35 °C. The mobile phase composed of (A) H_2O–acetic acid (100:1.5, v:v) and (B) methanol with a gradient profile of 57–71 % (B) in 25 min. At 35 min, the mixture began to change to its initial composition. The column was equilibrated for 10 min prior to each analysis. The flow rate was 1 mL/min. The UV spectra were taken in the region of 200–400 nm. The effluent was monitored at 330 nm by a PDA.

HPLC/ESI–MS analysis

The HPLC/ESI–MS analysis was carried out using Agilent 1100 series LC/MSD Trap (Agilent, USA). The chromatographic conditions were the same as that for HPLC–PDA. ESI experiments were carried out in the positive mode. Drying nitrogen was heated to 150 °C and introduced into the capillary region at a flow rate of 10 L/min. The pressure of nebulizing nitrogen was set at 40 psi. The capillary temperature was kept at 250 °C, and the mass range measured was 100–1,000 *m/z*.

NMR analysis

^{1}H and ^{13}C-NMR spectra were recorded in [$^{2}H_1$] chloroform (CDC_{13}) on a Bruker AV400 (Bruker, Switzerland) with 400 MHz for ^{1}H measurements and 100 MHz for ^{13}C measurements, respectively.

Antifungal testing

The MICs of the three PMFs obtained from our separation procedure were determined by using the microbroth dilution assay recommended by the Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) for filamentous fungi (M38-A) [17] with minor modifications. *A. niger* (CICC 2273) was purchased from China Center of Industrial Culture Collection and cultured for 5–7 days at 28 °C in potato dextrose agar. The PMFs were dissolved in 100 % dimethyl sulfoxide (DMSO) and diluted in RPMI 1640 medium buffered to pH 7.0 with 3-(*N*-morpholino)propanesulfonic acid (MOPS). And 180 μL of diluted sample solutions was dispensed into 96-well microdilution trays, and the final concentrations were in the range 3.2–0.025 mg/mL (0.025, 0.05, 0.1, 0.2, 0.4, 0.8, 1.6 and 3.2 mg/mL, respectively). Then, 20 μL of 10^4 CFU/mL (absorbance values of 0.09–0.11 at 530 nm) of inoculum suspension was inoculated onto microplates, and the test was performed in a volume of 200 μL. The same tests were performed simultaneously for growth control (RPMI + -fungi) and sterility control (RPMI). Plates were incubated at 35 °C for 48 h. The MIC was defined as the lowest concentrations preventing any discernible growth. The lowest MIC value represents the highest capacity for inhibition of antifungal activity.

Results and discussion

Isolation and identification of PMFs

Preparative isolation of the crude PMFs by HSCCC employed a two-phase solvent system composed of *n*-hexane–ethyl acetate–methanol–water (1:0.8:1:1, v:v:v:v) yielded three PMF components (Fig. 1). Then, 6.4 mg of compound 1, 20.2 mg of compound 2 and 2.8 mg of compound 3 were obtained from 70 mg of the crude PMFs by one-step HSCCC separation. The purity of them was 97.7, 99.1 and 95.9 %, respectively, as determined by HPLC area normalization method. The chromatograms of HPLC spectra of these compounds are shown in Fig. 2. Identification of the three compounds (Fig. 3) was carried out by physical properties, HPLC/ESI–MS and ^{1}H and ^{13}C-NMR spectra analysis as follows.

Compound 1 (NOB): Colorless needles; Mp 137–138 °C; ESI–MS: m/z 403 [M + H] + ; 1H NMR δ: 7.58(1H, dd, J = 8.4 and 2 Hz, H-6′), 7.42(1H, d, J = 2 Hz, H-2′), 7.00(1H, d, J = 8.4 Hz, H-5′), 4.11, 4.03(each 3H, s, OMe), 3.97(12H, m, 4 × OMe); 13C NMR δ: 177.4(C-4), 161.1 (C-12), 151.9(C-4′), 151.4(C-7), 149.3(C-3′), 148.4(C-5), 147.7(C-9), 144.1(C-6), 138.0(C-8), 124.0(C-1′), 119.6(C-6′), 114.9(C-10), 111.2(C-5′), 108.6(C-2′), 106.9(C-3), 62.3, 62.0, 61.8, 61.7, 56.1, 56.0(6 × OMe). Compound 1 was identified as 5,6,7,8,3′,4′-hexamethoxyflavone (NOB) from these spectral data and physical properties.

Compound 2 (TAN): Light yellow needles; Mp 50–51 °C; ESI–MS: m/z 373 [M + H] + ; 1H NMR δ: 7.89(2H, d, J = 9.2 Hz, H-2′, 6′), 7.03(2H, d, J = 9.2 Hz, H-3′, 5′), 6.63(1H, s, H-3), 4.11, 4.03, 3.89(each 3H, s, OMe), 3.95(6H, s, 2 × OMe); 13C NMR δ: 177.4(C-4), 162.3 (C-4′), 161.2(C-2), 151.4(C-7), 148.4(C-5), 147.7(C-9), 144.1(C-6), 138.1(C-8), 127.7(C-2′, 6′), 123.9(C-1′), 114.9 (C-10), 114.5(C-3′, 5′), 106.7(C-3), 62.3, 62.0, 61.8, 61.7, 55.5(5 × OMe). Compound 2 was identified as 5,6,7,8,4′-pentamethoxyflavone (TAN) from these spectral data and physical properties.

Compound 3 (5-DN): Yellow needles; Mp 144–145 °C; ESI–MS: m/z 389 [M + H] + ; 1H NMR δ: 12.56(1H, s, 5-OH), 7.62(1H, dd, J = 8.4 and 2 Hz, H-6′), 7.45(1H, d, J = 2 Hz, H-2′), 7.03(1H, d, J = 8.4 Hz, H-5′), 6.64(1H, s, H-3), 4.14(3H, s, OMe), 4.01(12H, m, 4 × OMe); 13C NMR δ: 183.0(C-4), 164.0(C-2), 153.1(C-4′), 152.5(C-7),

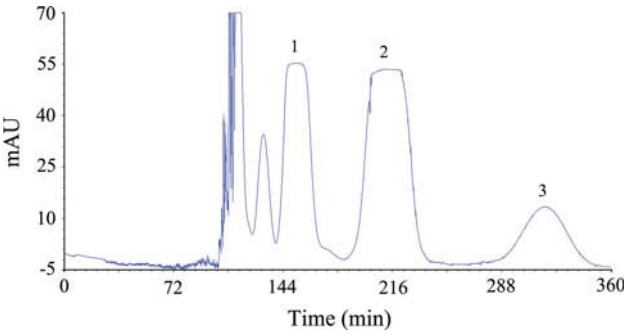


Fig. 1 HSCCC chromatogram of the crude PMFs *1*: nobiletin (NOB); *2*: tangeretin (TAN); *3*: 5-demethylnobiletin (5-DN)

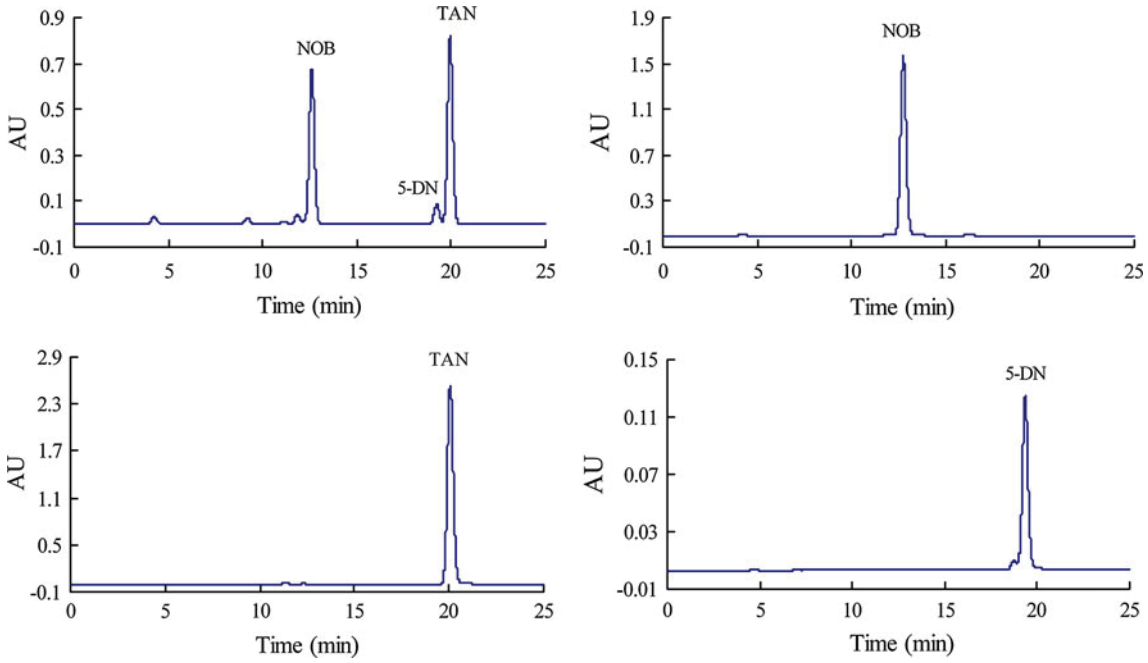
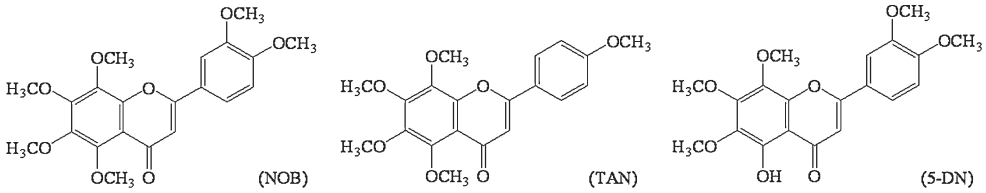


Fig. 2 HPLC chromatograms of the crude PMFs and compounds *1–3*

Fig. 3 The chemical structure of compounds *1–3*



149.6(C-3′), 149.4(C-5), 146.8(C-9), 136.6(C-6), 133.1(C-8), 123.7(C-1′), 120.2(C-6′), 111.3(C-5′), 108.8(C-2′), 107.0(C-10), 104.0(C-3), 62.1, 61.7, 61.2, 56.2, 56.0(5 × OMe). Compound 3 was identified as 5-hydroxy-6,7,8,3′,4′-pentamethoxyflavone (5-DN) from these spectral data and physical properties.

Antifungal activities of three PMF components from Ponkan

As NOB, TAN and 5-DN are three typical PMFs having comparable structural differences, they are ideal chemical compounds for the structure–activity relationship study. In the present study, we assayed the antifungal activities of the three PMFs from Ponkan peels against *A. niger*. The MIC values of NOB, TAN and 5-DN were 0.8, 0.4 and 0.1 mg/mL, respectively. From the MIC results and the chemical structure of PMFs, we can establish some interesting structure–activity relationships. 5-DN, monohydroxylated polymethoxyflavone with a 5-OH in the A-ring, displayed the best inhibitory effect on *A. niger*. When 5-OH was replaced by -OCH_3, 5-DN became NOB and the antifungal activity reduced, which suggested that 5-OH was an important functional group. Recently, 5-OH-PMFs have gained more attention, as considerable evidence suggests that 5-OH-PMFs have much stronger health-promoting biological activities than permethoxylated PMFs [18, 19]. The importance of 5-OH for the antibacterial activity of flavones and flavanones has also been reported by others [20, 21]. TAN exhibited stronger inhibitory activity than NOB, with the only structural difference between these two compounds being that NOB has a methoxyl group at position 3′ in the B-ring, while TAN has none. This indicates that 3′-OCH_3 in the B-ring reduced the antifungal activity of PMFs.

Conclusions

Three PMFs including NOB, TAN and 5-DN were successfully separated from Ponkan peel by HSCCC, and their chemical structures were identified by HPLC/ESI–MS and ^{1}H and ^{13}C-NMR. The antifungal activities of these PMFs against *A. niger* were evaluated, and the results showed some structure–activity relationships of PMFs: (1) A hydroxyl group at position 5 played a key role in the inhibitory activity,

and the exchange of 5-OH by -OCH_3 resulted in decrease in activity; and (2) the methoxyl group at position 3′ disfavored the inhibitory effect. The findings of this study suggest that PMFs have great inhibitory effects against *A. niger* and have enormous potential as natural preservatives. The results of this paper relating to antifungal structure–activity relationship of citrus PMFs are a good contribution in the field with potential applications.

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