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Structure–Activity Relationship of Citrus Polymethoxylated Flavones and Their Inhibitory Effects on *Aspergillus niger*

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ABSTRACT: Citrus peels are rich in polymethoxylated flavones (PMFs) and are potential sources of natural preservatives. Six PMFs extracts, isolated and purified from the peels of three mandarins (*Citrus reticulata*) and three sweet oranges (*Citrus sinensis*), were identified and quantitated. Their inhibitory effects on *Aspergillus niger* were evaluated using a microbroth dilution assay. The Red tangerine variety exhibited the greatest antifungal activity (MIC = 0.2 mg/mL), while Jincheng showed the lowest activity (MIC = 1.8 mg/mL). An analysis of principal components was applied to the results in order to elucidate the structure–activity relationships of the citrus PMFs. The structure–activity relationship analysis revealed that, for good inhibitory effect, the 5-OH, 3-OCH_3, and 8-OCH_3 functionalities were essential, while the presence of 3-OH and 3′-OCH_3 greatly reduced inhibition. The findings of this study provide important information for the exploitation and utilization of citrus PMFs as natural biopreservatives.

KEYWORDS: *Citrus reticulata, Citrus sinensis, polymethoxylated flavones, Aspergillus niger, antifungal activity, structure–activity relationship*

INTRODUCTION

Polymethoxylated flavones (PMFs) are a general term for flavones bearing four or more methoxyl 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 (*Citrus reticulata*) and sweet oranges (*Citrus sinensis*).[1] PMFs have recently been of particular interest due to their broad spectrum of biological activities, including antioxidant, anti-inflammatory, antiallergic, antiproliferative activities, and effects on mammalian metabolism.[1−6]

Recent studies have reported that PMFs are also potent inhibitors of microbial growth, possessing antibacterial,[7] antifungal,[8] and antiviral[9] activities. As a result of increasing consumer preference toward more natural and healthier products, scientific research has begun to focus on the screening of natural antimicrobial compounds as possible biopreservatives. The peels of citrus, which are rich in PMFs, are potential sources of natural preservatives preventing microbial growth, contributing to food safety and preservation. The antimicrobial activity of PMFs are directly related to their chemical structure, depending on the number and positions of hydroxyl groups and methoxyl groups.[10,11] Hydroxylation at position 5 is important for antibacterial activity and the presence of a lipophilic group at position 6 or 8 also improves activity.[12] Smejkal et al.[13] reported that methoxylation on the B-ring decreased antibacterial capacity, which correlated well with previous work by Alcaraz et al.[14] For C-ring, Mughal et al.[15] found that the replacement of the oxygen atom at position 4 with sulfur or nitrogen increased antibacterial activity. High antiviral activity was associated with the 4′-hydroxyl and 3-methoxyl groups, a substituent at the position 5 and a poly-substituted A ring.[16] Naturally occurring 4′-hydroxy-3-methoxyflavones possess antiviral activity against rhino- and polio-myelitis viruses.[3] Previous research has mainly focused on the antibacterial or antiviral activities of PMFs; however, information regarding antifungal capacity and structure–activity relationship of PMFs is lacking.

Aspergillus niger is a common fungal contaminant of food and some *Aspergillus niger* strains can produce mycotoxins harmful to human health. The presence and growth of most fungi could cause spoilage and result in a reduction in food quality. In this study, the antifungal activity of PMF extracts from the peels of six citrus varieties was studied against *Aspergillus niger*. Principal components analysis was performed aiming to reveal the structure–activity relationships of citrus PMFs as antifungal agents.

MATERIALS AND METHODS

Materials. Six typical citrus varieties cultivated in China were selected as raw material, including three mandarin, Ponkan (*C. reticulata* Blanco cv. Ponkan), Satsuma (*C. unshiu* Marc.), Red tangerine (*C. tangerina* Hort. ex Tanaka), and three sweet orange, Hamlin (*C. sinensis* Osbeck cv. Hamlin), Changyecheng (*C. sinensis* Osbeck cv. Changyecheng), and Jincheng (*C. sinensis* Osbeck cv. Jincheng) varieties. All varieties at mature stage were collected from the Yichang Citrus Experiment Station (Hubei, China) and Wangchunhua Citrus Valley Co. Ltd. (Hubei, China). The citrus peels were oven-dried at 40 °C to constant weight and milled into a powder (particle size 0.425 mm). The powder was placed in plastic bags and stored in the dark at 4 °C until used.

All chemicals in the investigation were of analytical grade, and were purchased from Sinopharm Chemical Reagent Co. (Shanghai, China),

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except for methanol, which was chromatographic grade (Fisher Chemical Co., USA) and used in HPLC and LC-MS/MS.

Standards of nobiletin (5,6,7,8,3′,4′-hetramethoxyflavone) and tangeretin (5,6,7,8,4′-pentamethoxyflavone) were purchased from Shanghai Yuanye Biotechnology Co., Ltd. (Shanghai, China) (purity >98%). Baicalein (5,6,7-trihydroxyflavone) standard was purchased from National Institutes for Food and Drug Control (Beijing, China) (purity >98.5%).

Apparatus. The high-speed countercurrent chromatography (HSCCC) system used for this work 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 HPLC system (Waters, USA), consisting of a 2695 separations module, a 2998 photodiode array detector (PDA), and an empower HPLC workstation, and Agilent 1100 series LC/MSD Trap (Agilent, USA) were used for analysis.

Extraction and Purification of PMFs. Approximately 100 g of citrus peel 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, freeze-dried with a yield of crude PMFs about 0.3−0.9 g.

The purification of crude PMFs was performed by HSCCC as previously reported by Wang et al.[17] The solvent system was composed of *n*-hexane−ethyl acetate−methanol−water (1:0.8:1:1, v/v), with the organic upper phase used as the stationary phase. The sample solution was prepared by dissolving 150 mg of crude PMFs in 15 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 effluent was continuously monitored by a UV detector at 340 nm. For each citrus variety, peak fractions were collected together, concentrated, and freeze-dried to yield purified PMFs extracts (purity >90%).

HPLC and LC-MS/MS Analysis of PMFs Extracts. The constituent and quantitative analysis of purified PMF extracts was determined by HPLC and LC-MS/MS. PMF extracts (1 mg) were weighed and dissolved in 10 mL of methanol (HPLC grade); 20 μL of the sample solution was injected.

For HPLC experiments, the column used was a 250 mm × 4.6 mm i.d., 5 μm, Sepax Amethyst C18−H, the mobile phase was composed of H_2O−acetic acid (100:1.5, v/v) (A) and methanol (B). The linear gradient profile was 35−71% (B) in 65 min. At 75 min, the mixture began to change to its initial composition. The column was equilibrated for 15 min prior to each analysis. The flow rate was 1 mL/min. The UV spectra were taken in the region of 200−400 nm, and the PDA detector was set at 330 nm. The contents of nobiletin and tangeretin were determined by external standard methods, and other PMFs were determined by semiquantitative methods using baicalein as an internal standard.

LC-MS/MS identifications of PMFs were performed on a system consisting of an ion trap mass spectrometer with an electrospray ionization (ESI) interface and LC system. The chromatographic conditions were the same as for HPLC. ESI experiments were carried out in the positive mode. Dry 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*. Peaks were identified by spectroscopic analysis with MS and UV−vis spectra comparing with authentic standards (nobiletin and tangeretin) and reference data.

Antifungal Testing. The minimal inhibitory concentration (MIC) of PMF extracts was determined by using a microbroth dilution assay as outlined by the Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) for filamentous fungi (M38-A) with minor modifications.[18] *Aspergillus niger* (CICC 2273) was purchased from the China Center of Industrial Culture Collection and cultured for 5−7 days at 28 °C using potato dextrose agar. Stock solutions of PMF extracts were prepared in dimethyl sulfoxide (DMSO) and diluted in RPMI 1640 medium buffered to pH 7.0 with 3-(*N*-morpholino)-propanesulfonic acid (MOPS). Then, 180 μL of diluted sample solutions were dispensed into 96-well microdilution trays providing final concentrations in the range of 2.6−0.1 mg/mL (2.6, 2.2, 1.8, 1.4, 1.0, 0.6, 0.2, and 0.1 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 of the PMF extracts was defined as the lowest concentrations preventing any discernible growth, that is no fungal mycelial growth. The lowest MIC value represents the highest capacity for inhibition of antifungal activity.

Statistical Analysis. Principal components analysis (PCA) was performed using SPSS 16.0. PCA was used in order to relate the antifungal activity of PMF extracts from six citrus varieties with their PMF composition.

RESULTS AND DISCUSSION

Constituent and Quantitative Analysis of PMFs Extracts. A total of eight components, including one flavonoid glycoside (compound **1**) and seven PMFs (compounds **2**−**8**), were indentified in the PMF extracts of six citrus varieties (Table 1). Figure 1 illustrates the chromatogram of PMF extracts from the Jincheng variety. These eight components were numbered according to their elution order. The positive ESI mass spectra showed only the molecular ions enabling the molecular weights of flavonoid glycoside and PMFs to be confirmed. Compound **1** was identified as flavone-7-*O*-[6-acyl]-glucoside, which is a acylated derivative from *O*-glycosyl-flavone. The fragment ion of $[M+H-162\text{-acyl}]^+$ from a global loss of the hexosyl and the acyl residues was observed.[19] Compounds **2**, **3**, **4**, **5**, and **8** were identified as tetra-, penta-, hexa-, and heptamethoxy-substituted flavones due to protonated molecular ions $[M + H]^+$ at *m*/*z* 343, 373, 403, and 433 in the positive mode. Compounds **6** and **7** could be assigned as being monohydroxylated hexa- or pentamethoxyflavone due to protonated molecular ions $[M + H]^+$ at *m*/*z* 419 and 389. The

Table 1. Structural Identification of Compounds 1−8

peak no.	t_R (min)	$[M + H]^+$ (*m*/*z*)	MS/MS (*m*/*z*)	UV-peak (λ/nm)	identification
1	31.7	543	283, 278	323, 255	flavone-7-*O*-[6-acyl]-glucoside
2	46.5	403	388, 373, 355, 266	335, 251	hexamethoxyflavone
3	51.7	343	328, 313, 299, 282	323, 264	tetramethyl-*o*-scutellarein
4	52.5	403	388, 373, 355, 342	335, 248	nobiletin
5	54.8	433	418, 403, 385, 354	344, 253	heptamethoxyflavone
6	57.3	419	404, 389	362, 255	natsudaidai
7	59.1	389	374, 359, 341, 328	344, 280	5-Demethylnobiletin
8	59.8	373	358, 343, 325, 312	326, 270	tangeretin

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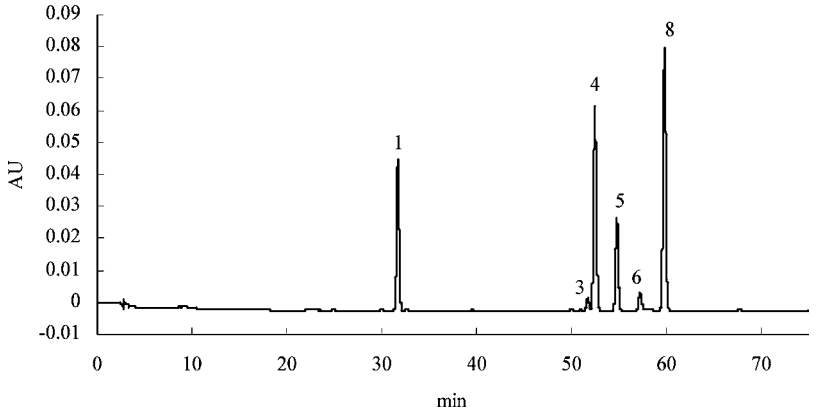
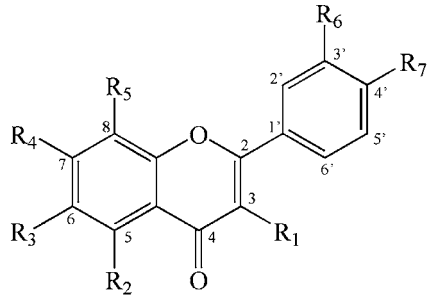


Figure 1. Chromatogram of Jincheng PMF extracts: 1, flavone-7-*O*-[6-acyl]-glucoside; 3, tetramethyl-*o*-scutellarein; 4, nobiletin; 5, heptamethoxyflavone; 6, natsudaidai; 8, tangeretin.



Peak no.	Compound	R1	R2	R3	R4	R5	R6	R7
2	Hexamethoxyflavone	OMe	OMe	OMe	OMe	H	OMe	OMe
3	Tetramethyl-*o*-scutellarein	H	OMe	OMe	OMe	H	H	OMe
4	Nobiletin	H	OMe	OMe	OMe	OMe	OMe	OMe
5	Heptamethoxyflavone	OMe	OMe	OMe	OMe	OMe	OMe	OMe
6	Natsudaidai	OH	OMe	OMe	OMe	OMe	OMe	OMe
7	5-Demethylnobiletin	H	OH	OMe	OMe	OMe	OMe	OMe
8	Tangeretin	H	OMe	OMe	OMe	OMe	H	OMe

Figure 2. Structures of identified PMFs in the extracts of six citrus varieties.

Table 2. Percentage (%) of Compounds 1−8 in the Extracts of Six Citrus Varieties

	varieties					
compounds	Ponkan	Satsuma	Red tangerine	Hamlin	Changyecheng	Jincheng
flavone-7-*O*-[6-acyl]-glucoside				0.79		22.76
hexamethoxyflavone		1.05		1.58	3.95	
tetramethyl-*o*-scutellarein	1.84	1.84	3.42	22.24	25.39	2.37
nobiletin	17.76	18.68	42.5	37.76	31.58	17.89
heptamethoxyflavone		60.53	6.18	24.74	26.32	15
natsudaidai		3.16				5.39
5-demethylnobiletin	2.5	0.66	1.71			
tangeretin	71.05	12.24	39.87	9.61	7.89	26.32

fragments of $[M + H - n \times 15]^+$ produced by the loss of one or more methyl groups from the protonated molecule, as well as $[M + H - 28]^+$, $[M + H - 33]^+$, $[M + H - 43]^+$, $[M + H - 46]^+$, and $[M + H - 61]^+$ fragment ions were diagnostic for the polymethoxylated species.[21] Compounds **1**−**8** were identified based on their UV spectra, molecular ions, fragment ions, and elution order described in the literature.[20−22] The structures and compound names of the identified PMFs in the extracts of citrus varieties are listed in Figure 2.

Composition and percentage content of PMF extracts from each citrus variety are listed in Table 2. Tetramethyl-*o*-scutellarein, nobiletin, and tangeretin were the main PMFs present in PMF extracts of the six selected citrus varieties, and their contents were much higher than hexamethoxyflavone, natsudaidai, and 5-demethylnobiletin. Heptamethoxyflavone was the most common PMF in five of the selected citrus varieties except for Ponkan; the Satsuma variety contained the highest heptamethoxyflavone content. 5-Demethylnobiletin was only detected in PMF extracts from three mandarin varieties

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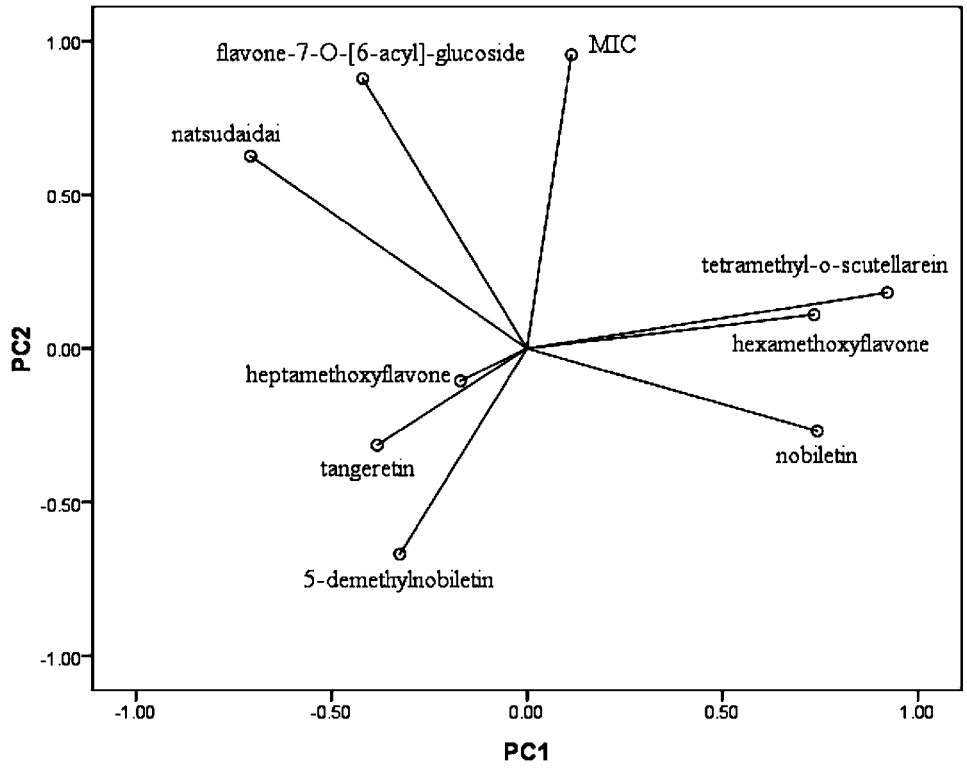

Figure 3. Plot of the principal components of the percentage (%) of PMFs and antifungal activity (MIC): PC1, 1st principal component; PC2, 2nd principal component.

and was not detected in the sweet orange varieties. It should be noted that the results reported in Table 2 indicate PMF contents of the extracts,and do not fully characterize the PMF content of citrus peels.

MIC Results. The MIC values of the PMF extracts from Ponkan, Satsuma, Red tangerine, Hamlin, Changyecheng, and Jincheng variety were 0.6, 0.6, 0.2, 1.0, 1.4, and 1.8 mg/mL, respectively. The Red tangerine variety exhibited the greatest antifungal activity (MIC = 0.2 mg/mL), while Jincheng showed the lowest activity (MIC = 1.8 mg/mL). The MIC values of mandarins were also lower than that of sweet oranges, indicating that the mandarin extracts had greater antifungal activity than sweet orange extracts. Higher MICs (≥1.6 mg/mL) were detected by Yi et al.[23] for PMFs against six strains of bacteria, which suggested that PMFs had better inhibitory activity against *Aspergillus niger* than some bacteria.

Structure−Activity Relationship Analysis. In order to elucidate the structure−antifungal activity relationships of PMFs, the MIC values were correlated with the percentages of PMFs through the analysis of principal components. The variables were the percentages of flavone-7-*O*-[6-acyl]-glucoside, hexamethoxyflavone, tetramethyl-*o*-scutellarein, nobiletin, heptamethoxyflavone, natsudaidai, 5-demethylnobiletin, and tangeretin. Three principal components were obtained from this analysis, of which the first two represented 76.6% of the total variance. PCA results are shown in Figure 3, and illustrate antifungal activities of individual PMFs. The graph of the two first components showed that the variables, 5-demethylnobiletin, tangeretin, and heptamethoxyflavone, contributed the most antifungal capacity to the PMF extracts, taking into account the inverse form of expressing antifungal activity (MIC). Flavone-7-*O*-[6-acyl]-glucoside and natsudaidai exhibited poor antifungal capacity, while an intermediate value was observed for nobiletin, hexamethoxyflavone, and tetramethyl-*o*-scutellarein.

In comparison with flavone-7-*O*-[6-acyl]-glucoside, the seven PMFs showed higher antifungal activity; this finding is in agreement with previous studies about antibacterial and antiviral activities of PMFs. In this respect, Manthey et al.[24] found that some PMFs strongly inhibited bacterial lipopolysaccharide-induced expression of TNF-α, whereas flavonoid glycosides were inactive. The antiviral activity also associated with nonglycosidic compounds.[11] It was suggested that glycosides were hardly permeable to cell membranes and could not reach the active sites, therefore, showed less activity.[25] PMFs occur in nature without glycosidic linkages, which may increase their permeability to cell membranes of microorganisms and enhance their antimicrobial properties.

From PCA results shown in Figure 3, 5-demethylnobiletin, monohydroxylated polymethoxyflavone with a 5-OH in the A-ring, displayed the best inhibitory effects toward *Aspergillus niger*. When 5-OH was replaced by $-OCH_3$, 5-demethylnobiletin became nobiletin, and the antifungal activity reduced, which suggested that 5-OH was an important functional group. The antibacterial activity of flavones and flavanones was also associated with 5-OH.[26] For chalcones, hydroxyl group at position 2′ (corresponding to position 5 of flavones) is considered a crucial group for structure stability, which could improve inhibitory activity, while methoxylation at position 2′ decreases antibacterial activity.[27] 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 because of the presence of 5-OH.[28,29] Another monohydroxylated polymethoxyflavone, natsudaidai, having a hydroxyl group at position 3, showed the lowest antifungal activity in the study. This suggested that the

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position of the hydroxyl group affects antifungal activity of PMFs. The hydroxyl group at position 3 was considered a prerequisite for antiviral activity of flavonoids,[11] while their antibacterial activity decreased in the presence of 3-OH,[30] as the mechanism of action could be different.

The antifungal activity of the other PMFs was determined as follows: tangeretin ≈ heptamethoxyflavone > nobiletin > hexamethoxyflavone ≈ tetramethyl-*o*-scutellarein. Heptamethoxyflavone exhibited stronger inhibitory activity than nobiletin, with the only structural difference between these two compounds being that heptamethoxyflavone has a methoxyl group at position 3 in the C-ring, while nobiletin has none. This indicates that the methoxyl group at position 3 in the C-ring contributes to the antifungal activity of PMFs. In comparison with other PMFs without 3-OH, natsudaidai exhibited the lowest antifungal activity indicating that 3-OH decreases the inhibitory effect, so it can be concluded that the order of potency for group substitution at position 3 is $-OCH_3$ > −H > −OH. The methoxyl group at position 8 also appears to be an important feature with regard to the antifungal activity of PMFs. Supporting evidence is from the fact that heptamethoxyflavone showed higher activity than hexamethoxyflavone, which had a similar structure except for the 8-OCH_3. Tangeretin, heptamethoxyflavone, and nobiletin, all having methoxyl group at position 8, showed higher antifungal activity than hexamethoxyflavone and tetramethyl-*o*-scutellarein without 8-OCH_3. The importance of 8-OCH_3 for antifungal activity appears to be similar for antibacterial and antiviral activity. Li et al.[12] reported that the presence of a lipophilic group at position 6 or 8 improves the antibacterial activity of flavonoids. Methoxyflavones, polysubstituted in the A-ring, show a higher antiviral activity than do monosubstituted compounds.[16] With a methoxyl group at position 3′, the inhibitory activity of nobiletin was poor, in comparison to tangeretin, which had the same structure but void of 3′-OCH_3. The 3′-OCH_3 in the B-ring reduced the antifungal activity of PMFs.

The structure−activity relationship analysis is helpful in understanding the mechanisms of PMFs in inhibition against fungi. Previous flavonoid research suggested that their antibacterial activity may be due to three main mechanisms: cytoplasmic membrane damage, inhibition of nucleic acid synthesis, and inhibition of energy metabolism.[10] Ortuno et al.[8] observed ultrastructural modifications in the hyphae when *Penicillium digitatum* was cultured in the presence of PMFs, indicating that the cell walls of the hyphae became thicker and a smaller cytoplasmic density was observed. This may be due to plasma membrane damage caused by perforation. The lipophilicity of PMFs could favor the interaction between PMFs and cell membrane. Therefore, the presence of the lipophilic substituents appears to improve antifungal activity of PMFs.

This research provides new information relating the chemical structure of PMFs to their antifungal activity. The results of this study provide information for the exploitation and utilization of PMFs as biopreservatives, and facilitate the design of chemical compounds with higher potency to serve as potential natural preservatives.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

PMFs, polymethoxylated flavones; HSCCC, high-speed countercurrent chromatography; MIC, minimal inhibitory concentration; PCA, principal components analysis

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