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Analytical Methods

Bioassay-guided isolation and identification of antifungal components from propolis against *Penicillium italicum*

S.Z. Yang [a,*], L.T. Peng [a], X.J. Su [b], F. Chen [c], Y.J. Cheng [d], G. Fan [a], S.Y. Pan [a,*]

[a] College of Food Science and Technology, Huazhong Agricultural University, Wuhan 430070, PR China
[b] Key Laboratory for Crop Germplasm Innovation and Utilization of Hunan Province, Hunan Agricultural University, Changsha 410128, PR China
[c] Department of Food Science and Human Nutrition, Clemson University, Clemson SC 29634, USA
[d] College of Horticulture and Forestry, Huazhong Agricultural University, Wuhan 430070, PR China

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ABSTRACT

The present study was aimed at identification of antifungal components against *Penicillium italicum* from Chinese propolis with bioassay-guided fractionation technique. Propolis ethanolic extract (PEE) was separated and purified by liquid–liquid extraction and thin layer chromatography (TLC) and the most active band was subjected to HPLC–MS/MS to identify the antifungal compounds. The results showed PEE and its fractions had strong antifungal activity against *P. italicum*. Among the fractions of PEE partitioned by petroleum ether, ethyl acetate, *n*-butanol and water, ethyl acetate fraction (E-Fr) exhibited the most effective activity against *P. italicum*. Further bioautographic TLC assay showed Band I, with Rf value of 0.70, had an inhibitive zone, which showed the strongest antifungal activity and completely inhibited the growth of *P. italicum* at 200 mg/L. Bioactive components found in Band I were further identified as pinobanksin, pinocembrine, chrysin and galangin. This study exhibited Chinese propolis and its main flavonoids was potential natural alternatives for the control of citrus blue mould caused by *P. italicum*.

1. Introduction

Blue mould, caused by *Penicillium italicum* Wehmer, is one of the most common fruit diseases occurring during storage and shipment of citrus (Montesinos-Herrero, del Rio, Pastor, Brunetti, & Palou, 2009). To prevent fruits decays, synthetic fungicides such as sodium *o*-phenylphenate (*o*-phenylphenol), thiabendazole and imazalil, are routinely used either preharvest or postharvest (Zhang, 2007). However, some of them have been removed from the market due to an increasing public concern regarding the contamination of fungicidal residues, as well as an increased resistance to fungicides in the pathogen populations (Kinay, Mansour, Gabler, Margosan, & Smilanick, 2007; Zhang, Zhu, Ma, & Li, 2009). Increased interest in the safe control of postharvest diseases of fresh fruits and vegetables has prompted the search for alternative methods for tradition disease control practices. One alternative could be use of natural products with antimicrobial properties, such as chitosan, jasmonates, glucosinolates, fusapyrone, essential oils and propolis (Raybaudi-Massilia, Mosqueda-Melgar, Soliva-Fortuny, & Martín-Belloso, 2009). Among these, propolis has been increasingly used as a natural preservative due to its efficacy in decreasing postharvest decay and extending the shelf-life of fruits and vegetables, including grapes, sweet cherries and citrus (Candir, Ozdemir, Soylu, Sahinler, & Gul, 2009; Min & Xiao, 2006).

Propolis, a resinous substance collected by honeybees from exudates and buds of the plants, contains various flavonoids, phenolic acids and their esters, sesquiterpenes, quinines, coumarins, steroids, amino acids, sugars, and proteins (Markham, Mitchell, Wilkins, Daldy, & Lu, 1996). Numerous evidences indicated that propolis has versatile pharmacological functions including antibacterial, antiviral, antifungal, anti-inflammatory, local-anaesthetic, antioxidant, antitumor and anti-helicobacter pylori activities (Siqueira et al., 2009). Propolis has a broad spectrum antimicrobial activity against bacterial species, yeast and several fungal species including phytopathogenic fungi like *Botrytis*, *Aspergillus*, *Alternaria Nees* (Campana et al., 2009; Silici, Koc, Ayangil, & Canaya, 2005; Umthong, Puthong, & Chanchao, 2009). The antimicrobial activities of propolis were supposed to be attributed to the presence of flavonoids or synergistic effects among its inherent phenolic components (Vardar-Unlu, Silici, & Unlu, 2008). More than 300 constituents have been identified in propolis sample, which indicated propolis was potential for new drugs (Sforcin & Bankova, in press). However, due to the complexity and variety of active constituents in propolis depending on its geographical and botanical origins, it was not easy to purify the functional components from propolis, and only several active components against clinical pathogens have been identified (Agüero et al., 2010). As far as we knew, few

* Corresponding authors.
E-mail addresses: yszhen@mail.hzau.edu.cn (S.Z. Yang), pansiyi@mail.hzau.edu.cn (S.Y. Pan).

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studies have been performed to test the antifungal property of propolis and its chemical composition against postharvest phytopathogens, especially *P. italicum*.

In our previous research, we found that propolis extracts showed strong inhibitory activity against green mould and blue mould of citrus fruits (Yang, Peng, Cheng, Chen, & Pan, 2010). The aim of this study focused on the identification of the antifungal components in propolis through chromatographic isolation and chemical characterisation by HPLC–MS, as well as synchronous antifungal bioassay against *P. italicum*.

2. Materials and methods

2.1. Propolis sample and fractionation

The crude propolis sample was collected from Baoding County, Hebei Province, China and stored at −20 °C for further usage. 5 g of frozen propolis sample was ground into fine powders and extracted with 200 ml of ethanol–water (80:20, v/v) at 40 °C for 4 h with slight agitation to obtain propolis ethanolic extracts (PEE). PEE was then fractionated using liquid–liquid extraction technique successively with petroleum ether, ethyl acetate, *n*-butanol and water to obtain four fractions, i.e., petroleum ether fraction (P-Fr), ethyl acetate fraction (E-Fr), *n*-butanol fraction (B-Fr) and water fraction (W-Fr). E-Fr was then separated by TLC using Silica gel 60 F_{254} as stationary phase and a solvent system composed of toluene:acetone = 3:1 as the mobile phase. The bands of TLC from E-Fr were respectively scraped from the dried plate and eluted with ethanol. The solvents of PEE and its subfractions were evaporated in vacuum and kept for antifungal activity evaluation.

2.2. P. italicum isolation

P. italicum was isolated from diseased citrus fruits with typical blue mould symptoms, and confirmed based on their morphological characteristics of conidiophores and conidia, and their growth features when incubated on healthy citrus fruits (Lu, 2001). The strain was cultured on PDA medium at 26 °C for 3 days, and then maintained at 4 °C until the next use.

2.3. Antifungal activity evaluation

The antifungal activities of PEE and its fractions were determined using the poisoned food technique (Perrucci et al., 1994). The freshly prepared and sterilized PDA medium was evenly distributed to several Petri dishes. One millilitre of PEE or its fraction solutions was mixed with 19 mL PDA, using the respective solvent as a control. Then three mycelial discs from 5-day-old fungal cultures were cultured in an equal space in the Petri dishes and incubated at 26 °C. For each treatment, three replicates were performed. The mycelial diameters were recorded after 72 h culturing and the mycelial inhibitive percentage was calculated by the following formula:

$$\text{mycelial inhibition percent} = \frac{(d_c - d_t)}{(d_c - d_i)} \times 100\%$$

Where d_c is the mean colony diameter of control sets; d_t is the mean colony diameter of treatment sets; d_i is the initial colony diameter of fungal PDA discs.

Effective concentrations for inhibition of 50% and 90% of mycelium radial growth (EC_{50} and EC_{90}) were calculated using the program DPS (Tang & Feng, 2002).

2.4. Bioautographic TLC assays

E-Fr from PEE was dissolved in ethanol to a final concentration of 1 mg/mL. An aliquot of 50 μL of the solution was spotted on a TLC plate (2.5 × 7.5 cm) and developed in the mixture solvents as mentioned above. Four bands were obtained from E-Fr, which were labelled as Bands I–IV. In the bioautographic assay, the treated TLC plate with separated chemical bands was covered by 18 mL of PDA medium containing 1×10^5 CFU/mL of *P. italicum*. The TLC plate was incubated overnight at 26 °C. The band exhibited the maximal inhibitive zone was considered the bioactive band, of which the Rf value was measured and calculated. Then the active band was collected and eluted with ethanol for further chemical analysis by HPLC–MS.

2.5. Chemical identification of the active fraction

Analyses of the chemical profile of the bioactive band was performed by an Agilent 1100 series HPLC–MS/MS system using an electric spray ionisation (ESI) interface (Agilent Technologies, Palo Alto, CA, USA). The HPLC system was installed with a 2.5 μm, 200 × 4.6 mm Hypersil ODS-C18 Symmetry analytical column maintained at 40 °C. An isocratic elution was performed with an aqueous mobile phase consisting of 65% methanol and 0.5% acetic acid at a flow rate of 0.8 mL/min with a running time of 20 min. The MS analysis was performed under the following condition: spay voltage, 3.0 kV; dry gas temperature, 300 °C; nebulizer pressure, 30.0 psi; sheath gas flow rate (nitrogen), 8 L/min; negative mode, scan range, m/z 100–1000.

3. Results

3.1. Antifungal activity of PEE on P. italicum

Antifungal activities of PEE on *P. italicum* were evaluated using the poisoned food technique. As shown in Table 1, the mycelia growths were inhibited in the presence of PEE, and the inhibitory efficiency were enhanced with the increment of PEE concentration. After 72 h cultivation, the mycelia growths of *P. italicum* were nearly completely inhibited as PEE concentration 1200 mg/L. Toxicity regression analysis demonstrated that the inhibitive parameters of EC_{50} and EC_{90} of PEE were 144.8 and 820 mg/L, respectively. The results showed PEE had antifungal activity against *P. italicum*.

3.2. Antifungal activity of the fractions from PEE

In order to explore the active constituents in the propolis, PEE was further fractioned by chromatographic partition with different solvents. Simultaneously, antifungal activities of the fractions against *P. italicum* were assessed. As shown in Table 2, E-Fr showed the highest antifungal activity against *P. italicum*, with a fungal inhibition up to 100% at a concentration of 200 mg/L. The other

Table 1
Effects of concentration of PEE on the inhibition of *P. italicum* mycelium.

PEE concentration (mg/L)	Colony diameter (mm)	Inhibition percent (%)
0	19.80 ± 0.76[a]	0
75	15.00 ± 0.63[b]	32.40
150	12.50 ± 0.55[c]	49.30
300	9.30 ± 0.52[d]	70.90
600	7.00 ± 0.45[e]	86.50
1200	6.00 ± 0.00[f]	93.20

Note: Each value of the inhibitive colony diameter was expressed in the means ± SD for three replicates after 72 h incubation. Different letters in the same column indicated significant differences at $P < 0.05$.

Table 2
Effects of 200 mg/L of different fractions from propolis ethanol extracts on the inhibition of *P. italicum* mycelium.

Fraction layer	Colony diameter (mm)		Inhibition percent (%)
	Control	Treatment	
W-Fr	19.67 ± 0.75[a]	18.67 ± 0.75[a]	6.82
B-Fr	13.00 ± 0.63[a]	11.00 ± 0.63[b]	25.00
E-Fr	14.33 ± 0.52[a]	5.00 ± 0.00[b]	100.00
P-Fr	14.67 ± 1.03[a]	11.25 ± 0.88[b]	35.40

Note: The value of each treatment was expressed in the means ± SD for three replicates after 72 h incubation. Different letters in the same line indicated significant difference at $P < 0.05$.

bioactive fractions showed their effectiveness in order of P-Fr and B-Fr. In contrast, W-Fr showed little inhibitory effect on the mycelia growth of the pathogen. Therefore, E-Fr was selected for further chemical separation and identification.

3.3. Bioautographic assays of E-Fr

E-Fr was separated by TLC and antifungal activities of the fractions were tracked by the bioautographic assay using *P. italicum* as the test strain. The results showed that the TLC offered four bands (Bands I–IV, as marked in Fig. 1a), Band I, with an Rf value of 0.70, exhibited a strong inhibitive zone, which indicated that Band I contained the active constituents against *P. italicum*.

3.4. Antifungal activity comparison of four bands from E-Fr

In order to further confirm the bioautographic results, antifungal activity of the four bands from preparative TLC were evaluated. All of the four bands exhibited inhibition on *P. italicum* at concentration of 200 mg/L (Fig. 2). Among them, Band I showed the strongest antifungal activity and completely inhibited the growth of *P. italicum* during the culture time (120 h), which was selected as the active band and prepared for HPLC–MS analysis.

3.5. Identification of antifungal active constituents

3.5.1. UV spectra of active constituents

Preliminary characterisation of flavonoids in Band I, which was separated and collected from the preparative TLC, was identified according to relevant colour reaction assays and their chemical UV spectra. The first chemical evidence resulted from the colour change of the solution when it became blue after addition of $NaBO_3$. A second chemical evidence came from spectral shifts of several special and characteristic wavelengths when the target chemicals were dissolved in methanol and methanol mixtures containing three different chemical-shift additives (AlCl3, NaAc, NaAc/HBO3), e.g., at UV λ_{max} = 291 nm (MeOH); 316, 395 (AlCl3); 329 (NaAc); 329 (MeONa); and 323 (NaAc/HBO3). According to the identification procedure introduced by Markham (1982), these characteristic wavelength of the solutions, which has a λ_{max} at 291 nm in methanol and shifts of 38 nm to the longer wavelength in MeONa or NaAc solution, are the characteristics of 5,7-hydroxy flavonoids. Therefore, it was preliminarily deduced that Band I possibly contained 5,7-hydroxy flavonoids as the bioactive constituents.

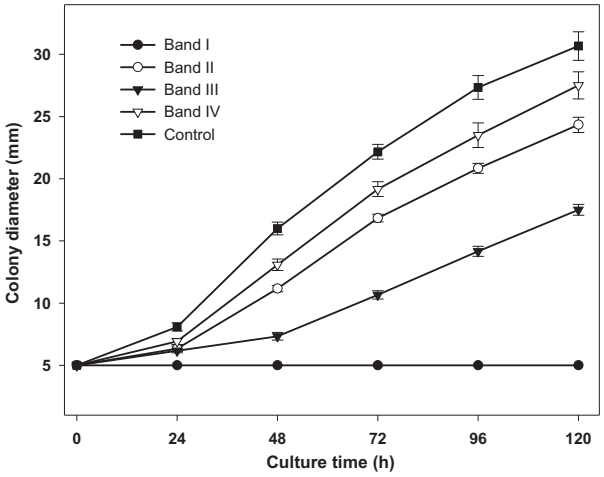

Fig. 2. Antifungal activities against the *P. italicum* of the four bands from the E-Fr collected by preparative TLC.

3.5.2. LC–MS/MS analysis of active constituents

As shown in Fig. 3, there were four major peaks in the LC chromatogram, corresponding to the retention time of 4.7, 7.1, 8.9, to 9.6 min, respectively.

3.5.2.1. Compound identification of Peak 1. The molecular weight of Peak 1 was 272, with its λ_{max} in the UV spectrum at 292 nm. The most characteristic fragments ions of Peak 1 obtained by MS^2 from its $[M-H]^-$ ions were m/z 253, 197 and 151. The fragment ion at 253 was formed by the neutral loss of H_2O ($[M-H-H_2O]^-$). Further loss of neutral fragment of CO from the parent ion of $[M-H-H_2O]^-$ ($[m/z]^-$ 253) led to the ion at $[m/z]^-$ 197 ($[M-H-H_2O-CO]^-$). The fragment ion at $[m/z]^-$ 151 was produced by a retro Diels–Alder cleavage from the ion of $[M-H-H_2O]^-$ ($[m/z]^-$ 253). Our LC–MS/MS data coincided with the data of pinobanksin (Gardana, Scaglianti, Pietta, & Simonetti, 2007; Gobbo-Neto, Gates, & Lopes,

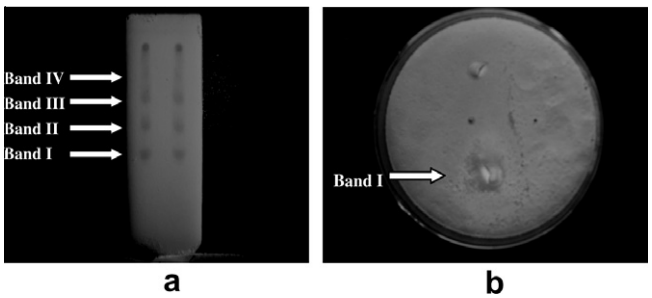

Fig. 1. TLC (a) and bio-autography against *P. italicum* (b) of the E-Fr from propolis ethanol extracts. The assay was carried out using Silica gel 60 F_{254} plate developed with toluene: acetone=(3:1). Fungal inhibition zone produced by the E-Fr is indicated with white arrow.

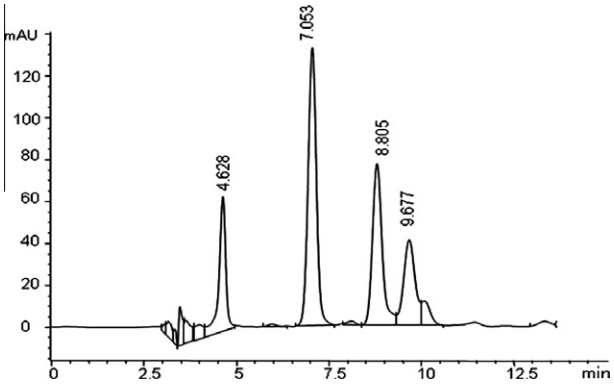

Fig. 3. HPLC chromatogram of active antifungal constituents in Chinese propolis.

2008). Therefore, Peak 1 was identified as pinobanksin, with the fragmentation explained in Fig. 4a.

3.5.2.2. Compound identification of Peak 2. The molecular weight of Peak 2 was 256, with its typical λ_{max} in the UV spectrum at 290 nm. The molecular negative ion of Peak 2 further produced some characteristic fragment ions by MS^2 with $[m/z]^-$ 213 ($[M-H-C_2H_2O]^-$) and $[m/z]^-$ 151 by a retro Diels–Alder from the ion of $[m/z]^-$ 255 ($[M-H]^-$) (Fig. 4b). These data were in agreement with the chemical characteristics of pinocembrine that was previously reported (Gobbo-Neto et al., 2008; Medana, Carbone, Aigotti, Appendino, & Baiocchi, 2008; Volpi & Bergonzini, 2006). So Peak 2 was identified as pinocembrine, with its fragmentation pattern shown in Fig. 4b.

3.5.2.3. Compound identification of Peak 3. The molecular weight of Peak 3 was 254. It has two λ_{max} peaks at 267 and 314 nm, which were in accordance with the UV spectra of chrysin (Gardana et al., 2007; Volpi & Bergonzini, 2006). As shown in Fig. 4c, the ion fragments of 225, 209, 153 were observed in the MS^2 spectra. Ions at 225 were formed by loss of CO from the ion of its molecular negative ion of 253, and the ion of 209 was formed by loss of CO_2 from the ion of 253. They were also present in the HPLC–MS spectra of chrysin from propolis (Gobbo-Neto et al., 2008; Medana et al., 2008; Nicolas, Isabelle, Edmond, & Joelle, 2001). Thus the Peak 3 was determined to be as chrysin.

3.5.2.4. Compound identification of Peak 4. The molecular weight of the compound of Peak 4 was 254, and its characteristic absorptive peaks in UV spectra were at 267 and 359 nm, which were in accordance with the UV spectrum of galangin (Volpi & Bergonzini, 2006). In its MS^2 spectra, the ion fragments of $[m/z]^-$ 241 ($[M-H-CO]^-$), $[m/z]^-$ 227 ($[M-H-C_2H_2O]^-$), $[m/z]^-$ 213 ($[M-H-2CO]^-$) were captured, which were the characteristic fragments of galangin from propolis (Medana et al., 2008). Compared with other previous analyses (Nicolas et al., 2001), the compound

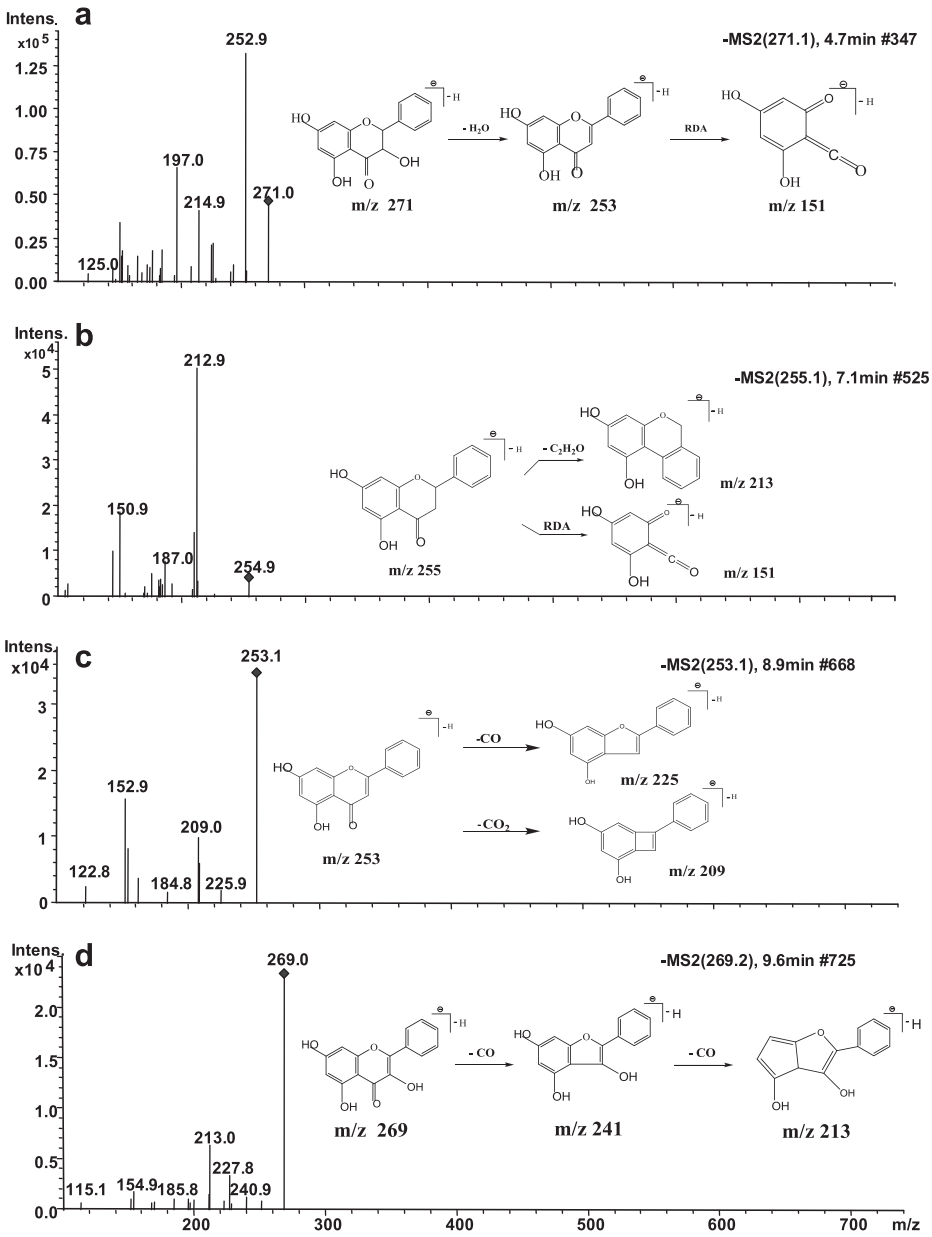

Fig. 4. Fragmentation patterns of active constituent from Chinese propolis. (a) Peak 1, pinobankson (b) Peak 2, pinocembrine (c) Peak 3, chrysin (d) peak 4, galangin.

of Peak 4 was identified as galangin. Its MS spectrum and fragmentation pattern are demonstrated in Fig. 4d.

4. Discussion

Propolis is a source of natural antibiotics and its ethanol extracts possess broad-spectrum inhibitory effects on bacteria, fungi, and nematode. In postharvest citrus disease control, propolis extracted by 70% ethanol completely inhibited conidial germination of *Penicillium digitatum* at all the tested concentrations, and 100 μg/mL propolis extract provided complete inhibition of naturally occurring green mould disease on wounded but uninoculated Star Ruby grapefruits (Soylu, Ozdemir, Erturk, Sahinler, & Soylu, 2008). Chinese propolis ethyl acetate extract showed strong antifungal activities against green mould and blue mould caused by *P. italicum* and *P. digitatum*, respectively (Yang et al., 2010). Here, the experiments demonstrated PEE and its fractions possessed significant inhibitory activity against *P. italicum*. Particularly, E-Fr and Band I from PEE exhibited strong antifungal activity against this pathogen, with complete inhibition at a concentration of 200 mg/L (Fig. 2). These results indicated Chinese propolis had great potential of a natural preservative that could be applied to control citrus postharvest disease.

The high antifungal activity of Chinese propolis against the pathogen led to investigate the corresponding active compounds. Phenolics and flavonoids were supposed the main antimicrobial components of propolis (Castro et al., 2009). Some flavonoids with strong antimicrobial activity have been identified from propolis collected from various regions. Pterocarpan medicarpin in Brazilian red propolis was a phytoalexin which demonstrated strong antifungal activity against Candida albicans (Fernandes, Lopes, Colombari, Monteiro, & Vieira, 2006). While in Turkey propolis the main antimicrobial compound was chrysin (Mercan et al., 2006) and in Bulgarian propolis galangin was the most active flavonoid against *Campylobacter jejuni* (Campana et al., 2009). Therefore, the antibacterial compounds in propolis could be qualitatively and quantitatively different with the variation dependent upon the region where it was collected. In Chinese propolis, as we investigated, the compounds possibly responsible for antifungal activities against *P. italicum* were identified as pinocembrine, pinobanksin, chrysin and galangin.

Some studies have demonstrated there was an important structure–activity relationship that influences antimicrobial activities of flavonoids, especially the position of OH groups, the properties of substituent groups, and hydrogen bond formation (Tripoli, Guardia, Giammanco, Majo, & Giammanco, 2007). Wang, Hamburger, Gueho, and Hostettmann (1989) demonstrated that the presence of hydroxyl group at C-5 and C-7 was very important for flavonoids activities against *Bacillus cereus*. Zheng, Tan, and Liu (1996) tested other methylated flavones which exhibited antibacterial activity mainly against Gram-positive bacteria, and showed that the most active were compounds with hydroxyl groups at C-5 and C-7 and with three substitutions in ring B. Of the 38 different flavonoids tested against strains of methicillin-resistant Streptococcus aureus, the active flavonoids have in common the obligatory C-4 keto group and hydroxyl group substitutions at C-3, C-5 and C-7 (Xu & Lee, 2001). The main compounds identified in Chinese propolis, namely pinocembrine, pinobanksin, chrysin and galangin, contained the critical groups of active flavonoids against bacteria, it was reasonable to expect their antifungal activity against *P. italicum*.

In conclusion, with the aid of chromatographic separation, identification and bioautographic assays of PEE and its separated chemicals, our experiments clearly demonstrated that PEE and its inherent flavonoids had strong antifungal activities against *P. italicum*, which indicated that the studied propolis had the potential to be a natural preservative that can be applied to control citrus postharvest diseases.

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