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Analytical Methods

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

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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

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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 $P < 0.05$.