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Use of active extracts of poplar buds against *Penicillium italicum* and possible modes of action

Shuzhen Yang [a], Limei Liu [a], Dongmei Li [b], Huan Xia [a], Xiaojun Su [c], Litao Peng [a,*], Siyi Pan [a]

[a] Key Laboratory of Environment Correlative Dietology, Ministry of Education, College of Food Science and Technology, Huazhong Agricultural University, Wuhan 430070, PR China
[b] Georgetown University Medical Center, Washington, DC, USA
[c] Hunan Provincial Key Laboratory of Crop Germplasm Innovation and Utilization, Hunan Collaborative Innovation for Utilization of Botanical Functional Ingredients, Hunan Agricultural University, Changsha, 410128, China

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ABSTRACT

Antifungal components, from poplar buds active fraction (PBAF) against *Penicillium italicum*, the causal agent of blue mold in citrus fruits, were identified and possible action modes were investigated. Pinocembrin, chrysin and galangin were determined as active components in PBAF, using HPLC and HPLC–MS analysis. The antifungal activity is stable at temperatures ranging from 4 °C to 100 °C and pH levels ranging from 4 to 8. In the presence of PBAF, the hyphae become shriveled, wrinkled and the cell membrane became seriously disrupted. Further investigation on cell permeability, nucleic acid content and alkaline phosphatase suggest that the cell membrane might be the target. Mycelial oxygen consumption and the respiration-related enzymatic activity of succinate dehydrogenase, malate dehydrogenase and ATPase were all inhibited by PBAF. We propose that PBAF is a potentially useful alternative for blue mold control and may act against *P. italicum* by interfering with respiration and disrupting the cell membrane.

1. Introduction

Blue mold, caused by *Penicillium italicum*, is one of the most common diseases that occurs in the storage and shipment of citrus fruits and causes substantial economic loss worldwide (Askarne et al., 2012). To prevent fruit decay farmers routinely apply synthetic fungicides, such as sodium orthophenylphenate, thiabendazole and imazalil, either at pre-harvest or post-harvest (Kinay, Mansour, Gabler, Margosan, & Smilanick, 2007). However, some of these fungicides have been banned due to increasing concern that fungicidal residue may contaminate the product and increase the likelihood of fungicidal resistance (Zhang, Zhu, Ma, & Li, 2009). The demand for the safe control of post-harvest diseases, in fresh fruits and vegetables, has prompted researchers to develop newer and safer antifungal agents.

Generally speaking, natural products extracted from plants represent a rich resource for screening bioactive compounds (Tripathi & Dubey, 2004). The buds of the genus *Populus*, widely distributed in temperate and subtropical regions, have been used traditionally as medicine to treat wounds (Bae, 1999; Van & Wink, 2004). Recent studies indicate that the exudates of poplar buds are the main resin source of poplar-type propolis, a popular folk medicine worldwide with broad pharmacological activities, exhibiting antibacterial, anti-inflammatory and cytostatic properties (Toreti, Sato, Pastore, & Park, 2013; Wilson, Spivak, Hegeman, Rendahl, & Cohen, 2013). The characteristic compounds in poplar buds excludes are flavonoids without B-ring substituents, such as pinocembrin, pinobanksin, galangin and chrysin, and phenolic acids and their esters, including caffeic acid phenethyl ester (CAPE), which has also been reported in propolis (Dudonne et al., 2011; Toreti et al., 2013). Furthermore, propolis exhibits the capacity to extend the shelf-life of grapes, sweet cherries, citrus and dragon fruits due to its effective antifungal activity (Noosheen, Asgar, Yasmeen, & Mehdi, 2013; Yang, Peng, Cheng, Chen, & Pan, 2010). As poplar bud exudates exhibit similar pharmacological activities to that of propolis (Dudonne et al., 2011; Wang et al., 2014), one can image that poplar bud extracts could have similar antifungal activities. However, the antifungal constituents of poplar buds and its mechanism of action against post-harvest fruit and vegetable pathogens, especially *P. italicum*, are still not well understood.

Metabolic respiration produces cellular energy, through the tricarboxylic acid cycle (TCA) and oxidative phosphorylation, used for cellular growth, multiplication, apoptosis and other processes of living cells in microorganisms (Thomas et al., 2014). The respiratory system is one of the most common targets for antimicrobial agents, including azoles and flavonoids including pinocembrin

* Corresponding author at: College of Food Science and Technology, Huazhong Agricultural University, Wuhan 430070, Hubei, PR China.
E-mail address: penglt12@mail.hzau.edu.cn (L. Peng).

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(Haraguchi, Tanimoto, Tamura, Mizutani, & Kinoshita, 1998; Peng et al., 2012; Sun et al., 2013). In addition, several studies have revealed that phenolic compounds containing hydroxyl groups could interact with the cell membrane of bacteria and cause leakage of cellular components (Xue, Davidson, & Zhong, 2013). In our preliminary study, crude poplar bud extracts exhibited strong antifungal activity to *P. italicum*. Here, the objective is to identify the active antifungal constituents of poplar buds and to investigate their possible antifungal mechanism through (i) the morphology of mycelia using scanning and transmission electron microscopy, (ii) membrane leakage, (iii) the oxygen consumption, and (iv) enzyme activities of the TCA pathway in mycelia of the pathogen.

2. Materials and methods

2.1. Fractionation of poplar bud and growth of P. italicum strain

Poplar buds (*Populus* × *euramericana cv.* '*Neva*') were collected from Heze City, Shandong Province, China. These buds were then ground into a powder and extracted successively with petroleum ether, dichloromethane, ethyl acetate, ethanol and water to obtain five fractions: i.e., petroleum ether fraction (PE-Fr), dichloromethane fraction (DM-Fr), ethyl acetate fraction (EA-Fr), ethanol fraction (E-Fr) and water fraction (W-Fr). After the antifungal assay, as described below, DM-Fr was separated further by column chromatography using macroporous resin AB-8 with 50%, 70%, and 90% ethanol as sequential eluents to obtain three fractions, so-called 50% fraction (Fr-1), 70% fraction (Fr-2), 90% fraction (Fr-3). All fractions were freeze-dried and stored at 4 °C for further analysis. Fr-2 was later named as the most active fraction of poplar buds (PBAF) since this portion demonstrated the strongest antifungal activity against *P. italicum*.

P. italicum was isolated from infected citrus fruits with the typical blue mold symptoms. The isolate was confirmed on the basis of the morphological characteristics of the spores, and the corresponding growth features when cultured on healthy fruit. The strain was cultured on potato dextrose agar (PDA) media at 26 °C and maintained at 4 °C after full growth. *P. italicum* spores were collected from one-week-old fungal cultures by scratching agar surface with a sterile glass rod. Spores were resuspended in distilled water containing 0.05% (v/v) Tween 80 and adjusted to 1×10^5 cfu/ml with sterile water.

2.2. Identification of the chemical constituents of PBAF

The chemical composition of PBAF was analyzed using a HPLC–DAD system (Waters Alliance E2695, USA) and an Agilent 1100 series LC–MS system with an electric spray ionization interface (Agilent Technologies, Palo Alto, CA, USA). The HPLC system was installed with a Hypersil ODS-C18 symmetry analytical column (2.5 μm, 200 mm × 4.6 mm) and 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 0.5 ml/min and an injection volume of 5 μl. The monitoring wavelength was set at 290 nm with a scan range of 200–800 nm. The compounds were identified on the basis of a combination of retention times and the spectra that matched standard compounds obtained from Sigma. MS analysis was performed under the following conditions: spray voltage, 3.0 kV; dry gas temperature, 300 °C; nebulizer pressure, 30.0 psi; sheath gas flow rate (nitrogen), 8 l/min; and negative mode, scan range, m/z 100–1000.

2.3. Spore germination and antifungal stability assays

In spore germination assays, 0.5 ml of the appropriate concentration of each poplar bud extract was added to 4.5 ml of 2% PDA tube maintained at 50 °C. The medium was immediately poured onto a 7.5 cm sterile glass slide. After solidifying, each PDA slide was inoculated with 50 μl of fungal spore suspension, placed on a 9 cm Petri dish with wet filter paper at the bottom, and incubated at 26 °C. After 12 h of incubation, approximately 50 spores per slide were examined under a Leica DME binocular light microscope (Leica Microsystem Inc, Germany). Each extract sample was performed in triplicate. The number of germinated spores with germ tubes longer than twice the diameter of the corresponding spores was counted and the inhibitory percentage of spore germination was calculated.

The antifungal stability of PBAF was determined by the spore germination inhibition assay after the sample was exposed to different temperatures and pH. Aliquots (10 ml) of PBAF solutions were treated at 25, 35, 60, 80, or 100 °C for 30 min. The samples were cooled down before the evaluation of antifungal activity. To investigate stability under the influence of pH, 1 ml of PBAF solution was mixed with 9 ml of PDA medium (the final concentration was maintained at 400 mg/l) and the pH of the mixture was adjusted to 3.0, 4.0, 5.0, 6.0, 7.0 and 8.0, respectively, with phosphate buffer. PDA media with same pH ranges was used as a control.

2.4. Effect of PBAF on mycelial growth of P. italicum

The effects of PBAF on the mycelial growth of *P. italicum* were analyzed using the method described previously by Yang et al. (2010) with slight modifications. In short, 19 ml of sterilized PDA medium was mixed with 1 ml of the appropriate concentration of PBAF (80% ethanol as solvent) and then poured into Petri dishes. Three mycelial discs (6 mm) from two-day-old fungal cultures were transferred on the PDA medium with PBAF in an equal space on the Petri dish and incubated at 26 °C. Each sample with different respective concentrations of PBAF was performed in triplicate. Colony diameters were measured after 72 h to record mycelial growth, and the mycelial inhibition rate was calculated using the following equation:

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

where d_c is the mean colony diameter of the control sets, d_t is the mean colony diameter of the treatment sets, and d_i is the initial colony diameter of fungal PDA discs.

2.5. Microscopic changes of P. italicum hyphae

The hyphae of *P. italicum* treated with active extracts were evaluated using a JSM-6390 scanning electron microscopy (SEM). 400 μl of spore suspension (10^4 spore/ml) was mixed with 15 ml PDA medium. 3–4 coverslips (25 mm × 25 mm) were placed onto PDA surface and incubated at 26 °C. After 18 h, the coverslips with *P. italicum* mycelia were removed and treated with 800 mg/l PBAF for 3 h at 26 °C. Afterward, the coverslips with *P. italicum* were washed three times with PBS, fixed with 2.5% (v/v) glutaraldehyde for 48 h, and washed three times with 0.05 mol/l phosphate buffer (pH 6.8) at 4 °C. The coverslips were subsequently dehydrated in a graded ethanol series (30%, 50%, 70%, 95% and 100%, v/v) for 20 min each and dried to the critical point in liquid CO_2. Mounted samples were sputter coated with palladium–gold and then examined under the SEM.

For the transmission electron microscopy (TEM) study, the mycelia from 48 h growth in potato dextrose broth (PDB) at 25 °C were treated with 800 mg/l PBAF on a gyratory shaker at 120 rpm for 3 h. The mycelia without PBAF treatment were used as a control. After treatment, the samples were harvested, washed

twice with distilled water, and pre-fixed in a 2.5% glutaraldehyde solution adjusted to pH 7.4 with 0.1 M phosphate buffer. The samples were fixed with 2% OsO_4 in the same buffer, dehydrated, and embedded in epoxy resin and SPI-812. Ultrathin sections, obtained with a Leica UC6 ultramicrotome, were stained with uranyl acetate and lead citrate. The stained sections were studied under a transmission electron microscope (H-7650, Hitachi, Ltd, Tokyo, Japan) at 80 kV and images were recorded using a CCD camera (Gatan Inc, Warrendale, PA, USA).

2.6. Cell wall and cell membrane integrity assays

The mycelia incubated in PDB at 26 °C for 48 h were treated with 800 mg/l PBAF at 26 °C on a gyratory shaker at 120 rpm. The mycelia without PBAF treatment served as a control. To determine the effect of PBAF on the cell wall, the activity of alkaline phosphatase (AKP) in the crude cellular extracts were measured using an assay kit (Nanjing Jiancheng Institute of Bioengineering, Nanjing, Jiangsu, China) following the manufacturer's instructions. The result was recorded as U/mg protein.

The degree of cell membrane injury can be easily estimated by measurement of electrolyte leakage and nucleic acid content. After 48 h of growth in PDB at 26 °C, 1 g of fresh mycelium were added into a sterilized conical flask containing 100 ml of 800 mg/l PBAF solution and cultivated as described above, mycelia unexposed to PBAF were used as control (Peng et al., 2012). Initial electrolyte leakage was determined by an electrical conductivity meter (model DDS-11A; Shanghai Scientific Instruments, Shanghai, China). Total electrolyte leakage was determined at 25 °C for each mycelia sample that was boiled for 10 min to kill the cells and release all the electrolytes completely before measurement. The relative leakage was calculated as followed formula:

$$\text{Relative leakage} = \frac{(P_1 - P_0)}{(P_k - P_0)} \times 100\%$$

where P_0 represents the ionic conductivity without mycelia; P_1 is ionic conductivity with mycelia; P_k represents ionic conductivity with boiled mycelia.

For nucleic acid content, 3 g of fresh mycelia were added into a sterilized conical flask containing 100 ml of 800 mg/l PBAF solution as described above. The samples were obtained every 4 h during a 12 h incubation period. 0.5 g of fresh sample was ground with liquid nitrogen and diluted to 100 ml with 0.1 mol/l sodium phosphate buffer (pH 7.0). After centrifugation at 12,000*g* for 15 min, the supernatant was used to determine the concentration of nucleic acid by measuring the absorbance at 260 nm (Paul, Dubey, Maheswari, & Kang, 2011).

2.7. Influence of PBAF on the oxygen consumption of P. italicum

The oxygen consumption rate (OCR) of the mycelia was determined at 20 °C with a Clark-type electrode (Hansatech Instruments, Norfolk, UK). After 48 h of growth at 26 °C, the mycelia were collected by vacuum filtration and washed with physiological saline thrice to remove medium residue. The wet mycelia were suspended in 20 mmol/l HEPES-TRIS buffer (containing 250 mmol/l mannitol, 10 mmol/l KCl, 5 mmol/l EDTA, and 20 mmol/l $MgCl_2$, pH 7.2) for further use. The mycelial suspensions were treated with appropriate amounts of PBAF at final concentrations of 200, 400 and 800 mg/l; the samples were stored for 30 min before oxygen consumption assays were carried out.

2.8. Succinate dehydrogenase (SDH), malate dehydrogenase (MDH), and ATPase assays

A crude enzyme solution was prepared by suspending 1.5 g of wet mycelia cultured for 48 h in 50 ml of PDB containing 800 mg/l PBAF; the suspended mycelia were collected after 0, 4, 8 and 12 h of culture at 26 °C on a gyratory shaker at 120 rpm. The mycelia were washed with distilled water twice and then centrifuged at 10,000 rpm for 10 min at 4 °C. The pellets were resuspended in 5 ml of 0.1 mol/l phosphate buffer (pH 7.4, containing 0.05% Triton X-100) and then treated to ultrasonication in an ice bath for 10 min. The cell debris was then centrifuged at 4 °C and the supernatant was used to measure enzyme activity. The protein content in each supernatant was determined using a Bio-Rad assay method (Bradford, 1976).

MDH, SDH, and ATPase activities were analyzed using an assay kit (Nanjing Jiancheng Ins., China) according to the manufacturer's instructions. One unit of MDH activity was defined as the amount of enzyme that causes a $0.01\Delta A_{340}$ increase in 1 min at 25 °C. One unit of SDH activity was defined as the amount of enzyme that reduces 1 μmol dichlorophenol-indophenol in 1 min at 25 °C. One unit of ATPase activity was defined as the amount of enzyme resulting in a hydrolysis rate equal to 1 μmol Pi/mg protein in 1 h.

2.9. Statistical analysis

Statistical analysis was performed using SPSS version 9 for Windows (SPSS Inc. Chicago, IL, USA). Each experimental procedure was conducted triplicates. Quantitative data were expressed as mean ± SD unless otherwise indicated. $P < 0.05$ was considered significant.

3. Results

3.1. Antifungal activity of poplar buds and chemical constituent analysis

To obtain the fraction with the highest antifungal activity, poplar buds were successively extracted with five different polar solvents and afterward the antifungal effectiveness of each fraction was assessed against *P. italicum* (Fig. 1a and b). The results showed that the DM-Fr exhibited the highest antifungal activity, with a fungal inhibitory percentage up to 60% at the concentration of 800 mg/l. The second and third bioactive fractions were EA-Fr and E-Fr. On the other hand, the PE-Fr showed only a mild inhibitory effect and W-Fr had no effect on spore germination. Thereafter, we focused on D-Fr for further extraction and purification. This portion was separated by column chromatography using macroporous resin, AB-8, with 30%, 50%, 70%, and 95% ethanol as eluents. The following antifungal assay confirmed that the 70% ethanol fraction (Fr-2) exhibited the most complete activity against *P. italicum* with a 100% inhibition rate at a concentration of 800 mg/l. and was denoted as PBAF (active fraction of poplar buds) for convenience.

The chemical composition of PBAF was analyzed by HPLC–DAD and LC–MS. As shown in Fig. 2 and Table 1, three main peaks were detected, in which Peak 1 yielded a UV λ_{max} at 290 nm with a retention time of 11.629 min and a molecular weight of 256; Peak 2 reached a UV λ_{max} of 267.4 and 313.8 with a retention time of 14.797 min and a molecular weight of 254; and Peak 3 had UV λ_{max} at 255 and 357.9 with a retention time of 16.781 min and a molecular weight of 270. Referring to the chemical characteristics that have been described previously, as well as comparison to authentic standards (Yang et al., 2011), the active constituents of PBAF were

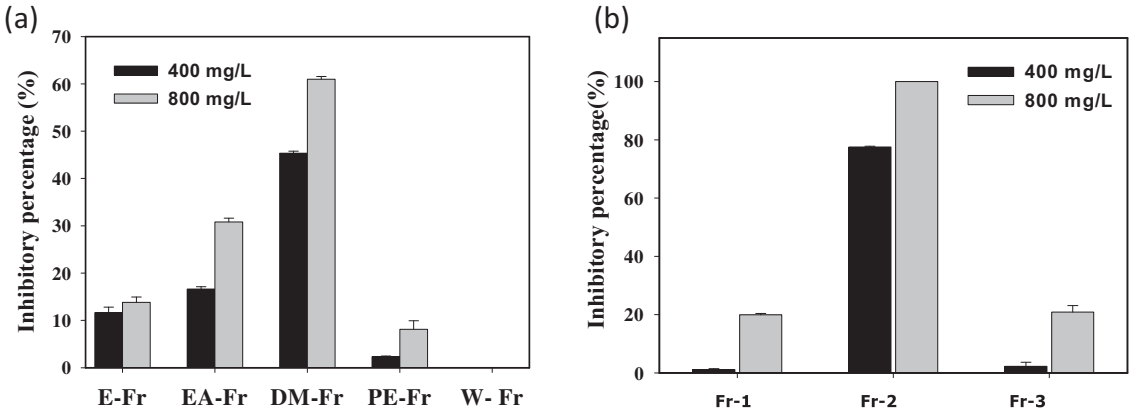

Fig. 1. Bioassay of poplar bud extracts against *P. italicum* and identification of its active chemical constituents. (a) Different poplar bud extracts; (b) different fractions of dichloromethane extracts separated by AB-8 resin.

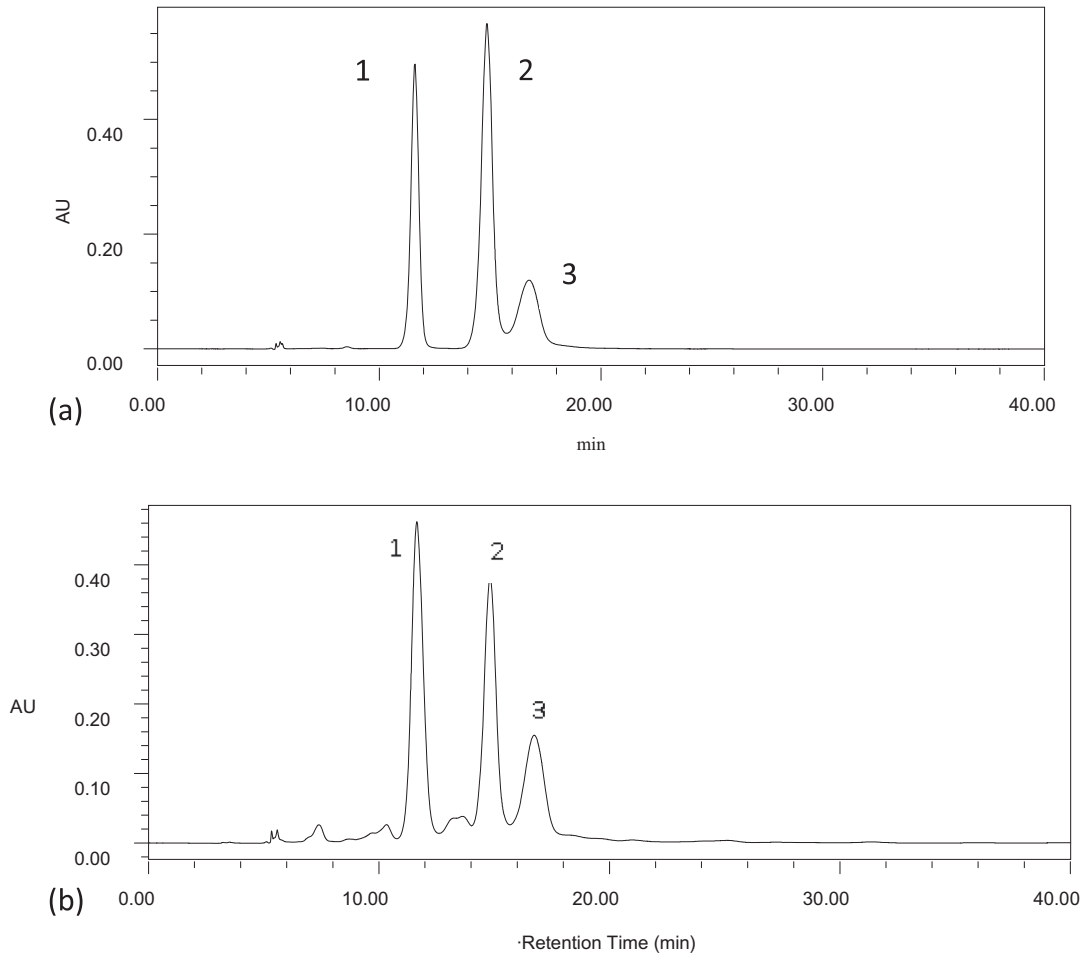

Fig. 2. Chromatograph of HPLC profile of PBAF at 280 nm. ((a) HPLC of mixed standards; (b) HPLC of PBAF; Peak 1, pinocembrin; Peak 2, chrysin; Peak 3, galangin.)

identified as pinocembrin for Peak 1, chrysin for Peak 2, and galangin for Peak 3.

3.2. Antifungal properties of PBAF against P. italicum

The antifungal sensitivity of PBAF against *P. italicum* was evaluated in the presence of PBAF as shown in Fig. 3. At 100 mg/l, the inhibition of spore germination was 13.5% but increased to 75% at 200 mg/l. As the dose increased, the inhibition of spore germination increased, until no germination was seen at 800 mg/l PBAF (Fig. 3a). For the stability of these compounds, we tested PBAF antifungal activity at temperatures from 4 °C to 60 °C, and the results showed that antifungal activity remained at over 60% when the temperature reached 100 °C (Fig. 3b). At the same time, PBAF also elicit strong inhibitory effects at pH values from 4 to 8, with more than 90% inhibitory effects remaining. However, this bioactivity decreased when PBAF treatment was administered at pH 3.0, even though he inhibition rate remained at 79.64% (Fig. 3c).

As shown in Fig. 3d, the mycelial growth was also affected in the presence of PBAF at different concentrations, indicating that a

Table 1
LC-ESI/MS data for the components of PBAF.

	Retention time (min)	Exact mass	Major ions m/z For $[M-H]^-$	UV λ_{max} (nm)	Compound identified
Peak 1	11.629	256.4	255.4	290.0	Pinocembrin
Peak 2	14.797	254.5	253.5	267.4, 313.8	Chrysin
Peak 3	16.781	270.5	269.5	255.0, 357.9	Galangin

concentration dependence also occurs for mycelial inhibition. Within 48 h of culture with 800 mg/l PBAF, the mycelial discs showed no signs of growth. However, this inhibitory effect reduced as incubation time was extended to 96 h.

3.3. Effect of PBAF on hyphal morphology and ultrastructure of P. italicum

The effects of PBAF on the morphological characteristics of *P. italicum* were examined by SEM and TEM (Fig. 4a–h). The hyphae of the control sample showed regular and homogeneous morphological features with a constant diameter and a smooth external surface (Fig. 4a and c). By contrast, the hyphae exposed to PBAF became slender, shriveled, and wrinkled; the hyphae also lost linearity, showing some depressions on the hyphal surface (Fig. 4b and d).

The ultrastructural changes in the hyphae of *P. italicum* treated with PBAF were further evaluated by TEM. In the control cells, all of the organelles – including the nucleus, the cell membrane, the mitochondria, and the vacuoles – showed normal appearances and intracellular septum with a dense cytoplasm that adhered to the plasma membrane and the cell wall (Fig. 4e and g). In the PBAF-treated cells, we found serious damage to these structures, including disrupted cell membranes, leaked cytoplasmic contents (Fig. 4f and h), and an altered hyphal cell wall which was noticeably thinner than that of the control cells. The outline of the cell membranes also became unclear in the hyphae of *P. italicum*; the cytoplasmic organelles had disintegrated, and massive vacillation and even empty cavities appeared.

3.4. Effect of PBAF on cell wall and cell membrane integrity

Given the facts that the cell wall and the cell membrane of *P. italicum* were morphologically distorted by the PBAF treatment, mentioned above, we tested cell structural integrity with alkaline phosphatase (AKP) activity – a parameter indicating cell wall integrity, cell membrane permeability and nucleic acid content. As shown in Fig. 5, PBAF treatment significantly increased the relative electronic conductivity values and decreased the nucleic acid content in the hyphae of *P. italicum* (Fig. 5a and b); however, AKP activity was changed only slightly in comparison with the untreated fungal cells during 12 h (Fig. 5c). These results suggest that cell membrane permeability, rather than the cell wall itself, had been seriously disrupted.

3.5. Effect of PBAF on fungal respiration and TCA

The mitochondrial function of *P. italicum* hyphae was also investigated under treatment of PBAF (Fig. 5a–d). We found that the oxygen consumption rate of *P. italicum* declined as the PBAF dose

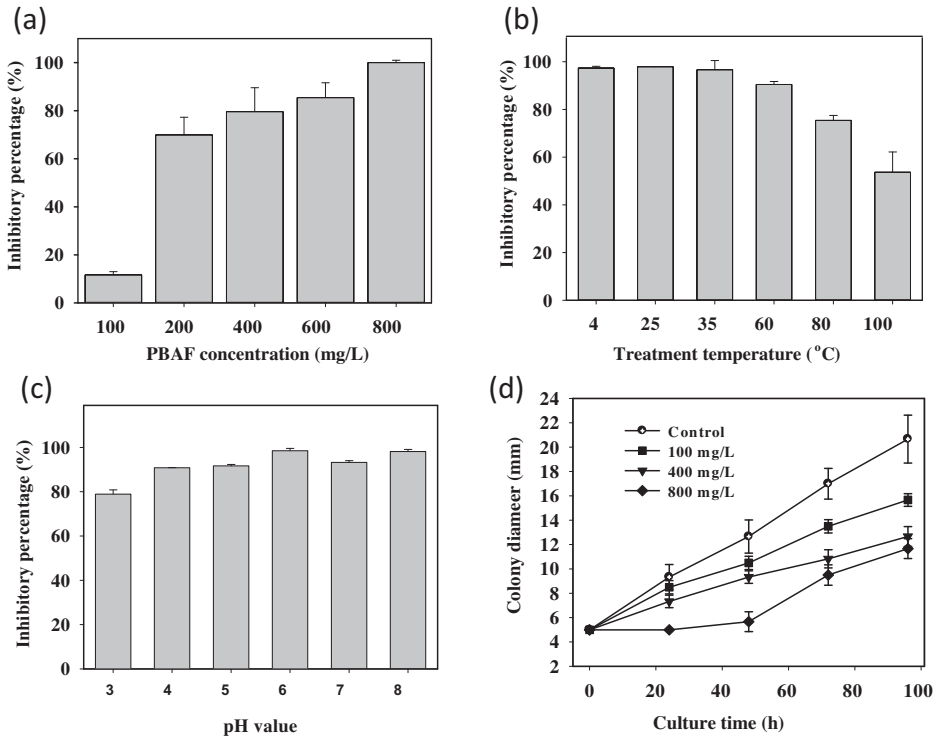

Fig. 3. Antifungal properties of PBAF on spore germination and mycelial growth of *P. italicum*. ((a) Spore germination inhibition; (b) antifungal stability of PBAF by different temperature treatments; (c) antifungal stability of PBAF under various pH; (d) mycelium growth inhibition.)

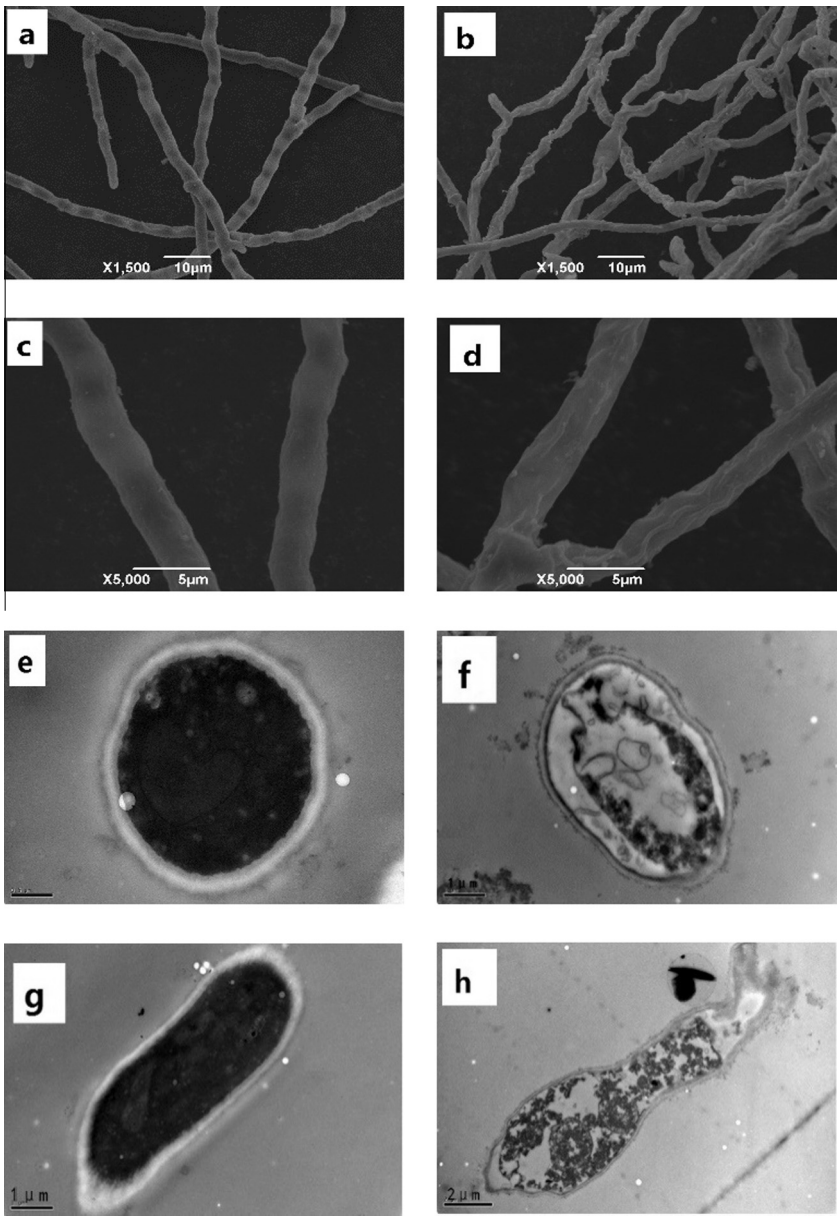

Fig. 4. Ultrastructural observation of *P. italicum* hyphae exposed to PBAF. ((a) SEM images of control hyphae (3000×); (b) SEM images of treated hyphae (3000×); (c) SEM images of control hyphae (5000×); (d) SEM images of treated hyphae (5000×); (e and f) TEM images of control hyphae; (g and h) TEM images of treated hyphae.)

increased. For example, the oxygen consumption rate was 0.018 nmol O_2/mg protein/min for 800 mg/ml PBAF treatment and 0.2 nmol O_2/mg protein/min for 200 mg/ml, in contrast to nearly 0.8 nmol O_2/mg protein/min without PBAF treatment (Fig. 6a). Furthermore, mitochondrial TCA enzyme activity – including MDH, SDH- and ATP hydrolysis enzyme ATPase – were altered under PBAF treatment. As shown in Fig. 5b and c, in the control mycelial cells the MDH activities was slightly elevated after 4 h of growth and then decreased somewhat, but remained high during 12 h of culture. This pattern was changed in PBAF-treated samples, where we found that the MDH activity was reduced remarkably at 4 h after treatment to 0.85 U/mg protein, which was only 32% of the MDH activity for the control (Fig. 6b). The SDH activity was also inhibited significantly by PBAF when compared with the control strain throughout the studied period (Fig. 6c). By the end, the entire cellular activity was downgraded by the decreased ATPase activity, as indicated by a mere 26% of control activity after 12 h treatment (Fig. 6d). In summary, these results suggest that PBAF depressed the metabolic activities of the hyphae by targeting the key enzymes of the tricarboxylic acid (TCA) cycle.

4. Discussion

Increasing awareness worldwide of health risks associated with synthetic fungicides has prompted researchers to explore new and presumably safer natural products for antimicrobial and food preservation agents (Goñi, Tomadoni, Moreira, & Roura, 2013). The effort to accomplish such a goal has identified a number of antimicrobial constituents derived from a series of poplar-type propolis and poplar tree buds, including chrysin, galangin, pinocembrin, quercetin, kaempferol and certain phenolic acids (CAPE, p-coumaric acid, etc.) (Bilikova, Popova, Trusheva, & Bankova, 2013; Peng et al., 2012; Yang et al., 2010, 2011). In the present study, we demonstrate that poplar bud extracts, especially

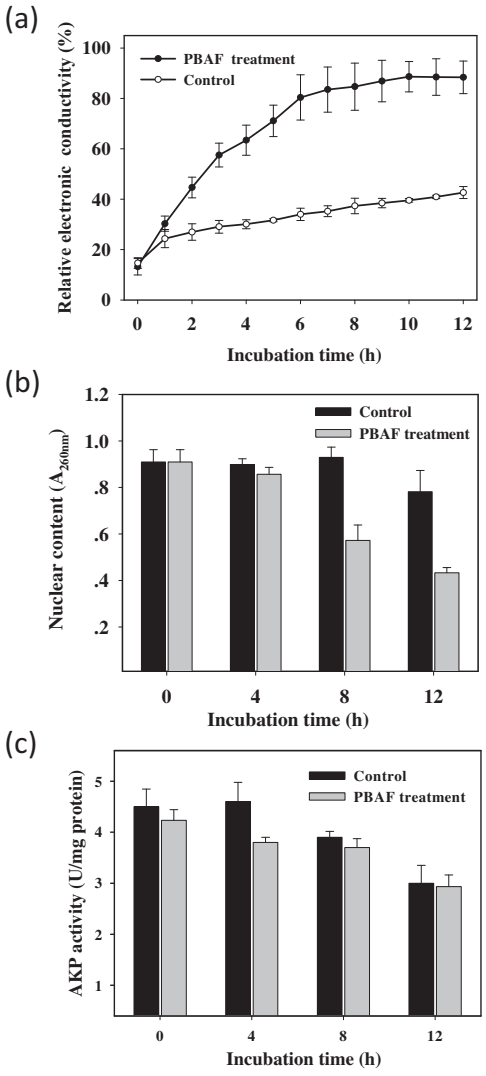

Fig. 5. Effects of PBAF incubation on cell membrane and cell wall integrity of mycelia of *P. italicum*. ((a) Relative electric conductivity. (b) Nucleic acid content. (c) Alkaline phosphatase activity of *P. italicum*.)

dichloromethane extract fractions, possess strong antifungal activity against *P. italicum* (Fig. 1a and b). Further investigation combined with a bioassay, HPLC and HPLC–MS confirmed that the flavonoids of pinocembrin, chrysin, and galangin are the active constituents in PBAF (Fig. 2 and Table 1). With evidence showing a stable antifungal activity at temperatures ranging from 4 °C to 100 °C and pH ranging from 3 to 8 (Fig. 3b and c), we conclude that PBAF can be potentially quite useful as a natural alternative to propolis for the control of citrus fruit spoilage.

Flavonoids extracted from plants have long been recognized for their antimicrobial activities. The antimicrobial mechanisms of flavonoids are generally associated with two main aspects: (1) the inhibition of nucleic acid synthesis, demonstrated by inhibiting DNA gyrase in *Escherichia coli* (Wu, Zang, He, Pan, & Xu, 2013), and (2) interference by flavonoids with the pathogens cytoplasmic membrane, thereby causing a change in membrane fluidity followed by outflowing of certain intracellular components (Cushnie & Lamb, 2005). However, the antimicrobial actions of this group of compounds are not limited to those noted above. For example, flavonoids alter the cell structure of pathogens, and epigallocatechin gallate inhibits biofilm formation and the viability of *Streptococcus mutans*. (Shumi, Hossain, Park, & Park, 2014; Xu, Zhou, & Wu, 2011). Others have reported that *Candida* spp. produce profusely flocculent extracellular material resembling a biofilm-like structure when exposed to baicalein, and later reduces *Candida parapsilosis* growth and cell viability (Serpa et al., 2012). When *S. aureus* is treated with sophoraflavanone B, a weakened cell wall and a damaged cell membrane were observed (Mun et al., 2014).

In the present study, morphological observations indicated that PBAF produced detrimental effects on the cell ultrastructure of *P. italicum* (Fig. 4). After exposure to PBAF, the hyphal cells lost their linearity, with depressions on the surface (Fig. 4a–d), the cell membrane became indistinct, and the cytoplasmic organelles disintegrated (Fig. 4e–h). Similar results have been observed in *P. italicum* treated with propolis or pinocembrin (Yang et al., 2011). Alkaline phosphatase (AKP) is produced in the cytoplasm and is normally secreted into the periplasmic space. When external cell wall damage occurs, the AKP enzyme will escape to the extracellular environment (Shao, Cheng, Wang, Yu, & Mungai, 2013). Consistent with the observation on the ultrastructure (Fig. 4e–h), we found AKP activity in PBAF-treated hyphae did not change markedly compared to that of the control group (Fig. 5c). It seems likely therefore, that the cell wall was not the main target of PBAF. Further investigations on cell membrane permeability and nucleic acid content of hyphae suggest that PBAF causes serious damage to the cell membrane (Fig. 4e–h). Perhaps, the cell membrane of *P. italicum* is one of the principle targets for PBAF.

Metabolic respiration is presumed to be the main energy-producing process for most microbial germination and growth. Also, the respiration system is one of the most common antimicrobial targets for flavonoids, such as licochalcone A, licochalcone C, and pinocembrin (Haraguchi et al., 1998; Peng et al., 2012), and azoles (Sun et al., 2013). Licochalcones A and C strongly inhibit oxygen consumption of Micrococcus luteus and *S. aureus* by targeting the site between CoQ and cytochrome c in the bacterial respiratory electron transport chain. In this study, the hyphae of *P. italicum* were sensitive to PBAF, which elicited concentration-dependent inhibitory effects on respiration (Fig. 6a); this result indicated that the respiration system of *P. italicum* may be one of the targets of PBAF that was also observed in our previous study with propolis and pinocembrin (Peng et al., 2012; Yang et al., 2010). The interference of respiration likely induces ATP depletion and triggers oxidative stress caused by further electron leakage. As a result, cellular components undergo oxidative damage (Li et al., 2011; Peng et al., 2012).

The tricarboxylic acid (TCA) cycle is coupled with energy production in the mitochondria and interacts with multiple anabolic pathways (Sierotzki & Scalliet, 2013). For example, MDH, one of the key enzymes in the TCA, performs important metabolic functions in aerobic energy-producing pathways and is also connected to malate shuttling (Yao, Li, Zhai, You, & Hao, 2011). In *Brucella abortus*, MDH is required not only for survival (Lowry et al., 2010) but also for pathogenesis. As a consequence, it has been considered as a virulence factor (Han et al., 2014). In our case, PBAF decreased MDH activity remarkably after 4 h of treatment (Fig. 6b), suggesting that the inhibitory effects of PBAF on the TCA cycle may lead to an imbalance between malate aspartate shuttle and reducing equivalents. Furthermore, another key enzyme in the TCA, SDH, is also involved with the oxidation of succinate to fumarate in the cytoplasm and reduce quinone to quinol in the membrane as it does in mitochondria (Hartman et al., 2014; Pecsi, Hards, & Ekanayaka, 2014). SDH is a regulator of respiration in *Mycobacterium tuberculosis* and is required for the maintenance of cell membrane potential under hypoxia (Pecsi et al., 2014). For pathogenic fungi, SDH is the target of fungicides, such as strobilurins and carboxamides (Leroux, Gredt, Leroch, & Walker, 2010). A recent study by Sierotzki and Scalliet (2013) showed that SDH mutations are found in 14 pathogenic fungal resistant strains. With

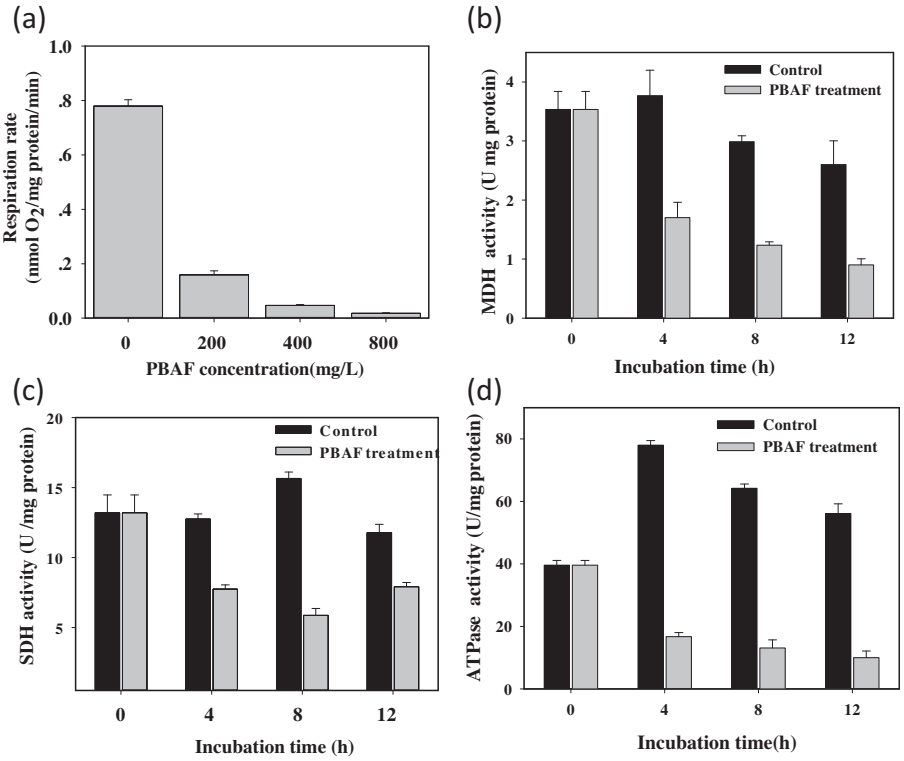

Fig. 6. Effects of PBAF on mycelia respiration and its relative enzymes of *P. italicum*. ((a) Respiration rate; (b) malate dehydrogenase (MDH); (c) succinate dehydrogenase (SDH); (d) ATPase.)

PBAF treatment, the SDH activity of *P. italicum* mycelia (Fig. 6c) resulted in a decrease of respiration and in the onset of a dysfunctional state that rendered the fungal cells unable to maintain membrane potentials, as indicated by the leakage of nucleic acids and an increase in relative electronic conductivity (Fig. 5a). In addition, as a marker of cell vitality, ATPases, a class of enzymes that catalyze ATP decomposition (Huang et al., 2014), were suppressed in PBAF treated cells (Fig. 6). We explain that this PBAF-inhibited ATPase limits the chances for fungal cell survivability, thereby eventually contributing to the inhibitory effect of PBAF on fungal growth. Although some reports showed that ATPases are involved in the resistance to antibiotics and fungicides and in the maintenance of mitochondrial homeostasis (Hot, Berthet, & Chesneau, 2014; Yang et al., 2014), the precise targets of PBAF remain unclear and warrant further investigation.

In summary, PBAF elicited strong and stable inhibitory effect on *P. italicum* through its antifungal components pinocembrin, chrysin, and galangin. PBAF treatment altered the morphological and ultrastructural characteristics of mycelia, caused damage to cell membrane permeability, induced the loss of nucleic acids, inhibited respiratory rate, and reduced respiration-related enzyme activities, including MDH, SDH, and ATPase. These results indicate that PBAF, perhaps, inhibits metabolic respiration and disrupts enzyme activities related to energy production in *P. italicum*. As a consequence, the cell membrane is disrupted and cell metabolism is impaired. Thus, PBAF effectively inhibited the mycelia growth of *P. italicum*. We expected that PBAF can be potentially useful as a natural alternative to propolis in the effort to control blue mold in citrus fruits.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2015.09.101.

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