Microwave irradiation enhances the *in vitro* antifungal activity of citrus by-product aqueous extracts against *Alternaria alternata*

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Running title: Antifungal activity of citrus waste extracts

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Summary

The effect of two lemon by-product aqueous extracts at different concentrations (14, 7, 3.5 and 1 mg mL^{-1}) were tested against the *in vitro* growth of *Alternaria alternata*. Prior to extraction one batch of by-product was dehydrated by freeze-drying (untreated by-product), while the other batch was treated by microwave irradiation in conjunction with freeze-drying (microwave treated by-product). High-performance liquid chromatography (HPLC) was employed for the identification of individual phenolic compounds with potent antifungal activities. Both lemon by-product aqueous extracts inhibited the mycelial growth and suppressed the spore germination of the fungus in a concentration-dependent manner. In general, the extracts obtained from the microwave treated lemon by-product displayed enhanced antifungal activity than those obtained from the untreated one. Scanning electron microscopy (SEM) revealed that both lemon by-product extracts affected the hyphal morphology of the fungus. The antifungal activity of the extracts was attributed to their phenolic acid and ascorbic acid contents.

Keywords: Mycelial growth inhibition; conidia; lemon waste; antifungal activity; phenolic compounds; sweet cherry.

Introduction

Alternaria alternata is a pathogen affecting both fruits and vegetables in the field and during storage (Feliziani *et al.*, 2013, Pane *et al.*, 2016). To date, this pathogen is mainly controlled by preharvest applications of synthetic fungicides (Feliziani *et al.*, 2013). However, the efficiency of some fungicides has been decreased due to the development of resistant strains of this pathogen (Avenot *et al.*, 2016). Moreover, pesticide residues might lead to environmental pollution and development of chronic diseases in humans (Kim *et al.*, 2017). Therefore, sustainable solutions for the control of pathogens implicated in fruit and vegetable decays should be explored.

Plant extracts contain a plethora of secondary metabolites which may be involved in plant defense mechanism (Tao *et al.*, 2014, Prakash *et al.*, 2015, Yang *et al.*, 2016, Pane *et al.*, 2016). Among the different classes of secondary metabolites polyphenols have an important contribution to the antifungal activities of the plant extracts (Wang *et al.*, 2017). Pane *et al.* (2016) reported that the foliar extracts of *Capsicum annuum* effectively controlled *A. alternata* growth on cherry tomatoes and this effect was attributed to the presence of polyphenols in the extracts.

A large amount of citrus by-products are annually generated by the juice industry and are a good source of polyphenols (Papoutsis *et al.*, 2017, Sharma *et al.*, 2017). Phenolic compounds of citrus peels include phenolic acids (Ma *et al.*, 2008), and flavonoids which are usually found in conjugated forms (Bilbao *et al.*, 2007). However, it is known that the free forms of phenolics are more active than the bound ones (Xu *et al.*, 2007, Wang *et al.*, 2017). Several studies have shown that the liberation of the bound polyphenols can be achieved by the application of heat or irradiation treatments on the dried materials (Hayat *et al.*, 2010, Kim *et al.*, 2008, Papoutsis *et al.*, 2016a, Papoutsis *et al.*, 2016d).

The aims of this study were: 1) to determine the bioactive profile of the aqueous crude extracts obtained from microwave treated and non-microwave treated lemon by-products with known antifungal activity against *A. alternata*, 2) to assess if microwave treatment of the freeze-dried lemon by-product can enhance the antifungal activities of the aqueous extracts, and 3) to assess growth patterns and identify morphological changes in the fungus hyphae using scanning electron microscopy.

Materials and methods

Materials

Lemon waste including peel, membranes and seeds, was kindly provided by a commercial juicing factory in Kulnura, NSW, Australia. After seed removal, the remaining peels and membranes with a moisture content of 85 ± 1% (mean ± standard deviation) were freeze-dried for 48 h as described by Papoutsis *et al.* (2017). The dried by-product was then ground using a commercial blender (Waring 2-speed blender, John Morris Scientific, Chatswood, Australia) and the powder passed through a 1.40 mm mesh sieve (EFL 2000; Endecotts Ltd., London, England) was used for the extraction. The dried lemon by-product was kept at −18 °C for further analysis.

Extract preparation

Microwave treatment of freeze-dried lemon by-product powder

Freeze-dried lemon by-product was treated according to Papoutsis *et al.* (2016a). Briefly, 1.5 g of freeze-dried lemon by-product was treated with 360 W for 5 min in a household microwave oven (1,200 W, frequency 2,450 MHz; Sharp Carousel, Abeno-ku, Osaka, Japan). After microwave treatment, the treated by-product was stored at −18 °C, until use.

Preparation of crude extract

Extraction was performed according to Papoutsis *et al.* (2016b) with some modifications. Briefly, 5 g of microwave treated or untreated freeze-dried lemon by-product powder was mixed with 100 mL of water and placed in a water bath (Labec Laboratory equipment Pty. Ltd., Marrickville, NSW, Australia) at 95 °C for 15 min. After extraction, the aqueous extracts were filtered using a Whatman no.1 filter paper to remove solid material. The filtrate was then transferred to a round bottom flask and concentrated under reduced pressure at 40 °C, using a rotary evaporator (Buchi Rotavapor B-480, Buchi Australia, Noble Park, VIC, Australia) before being freeze-dried for 48 h, in order to obtain the crude extract as a powder.

Phytochemical analysis of the extracts

Total phenolic content (TPC)

The TPC of the crude extracts was determined according to Papoutsis *et al.* (2016b). Briefly, 1 mL of crude extract was mixed with 5 mL of 10% (v/v) Folin–Ciocalteu reagent. After 3 minutes, 4 mL of 7.5% (w/v) Na_2CO_3 were added to the mixture and incubated in the dark at ambient temperature for 1 h. The absorbance of the solution was measured at 760 nm using a spectrophotometer (Varian Australia Pty. Ltd., Vic., Melbourne, Australia). The results were expressed as mg of gallic acid equivalents per g of dried extract (mg GAE (g d.e.)$^{-1}$).

Total flavonoid content (TF)

The TF of the crude extracts was determined according to Papoutsis *et al.* (2016b). Briefly, 2 mL of H_2O and 0.15 mL of 5% (w/v) $NaNO_2$ were mixed with 0.5 mL of sample and incubated at ambient temperature for 6 min. Then, 0.15 mL of 10% (w/v) $AlCl_3$ was

added and left at ambient temperature for 6 min. Subsequently, 2 mL of 4% (w/v) NaOH and 0.7 mL of H_2O were added and the mixture was incubated at ambient temperature for further 15 min. The absorbance was measured at 510 nm using a spectrophotometer (Varian Australia Pty. Ltd., Vic., Melbourne, Australia). The results were expressed as mg of catechin equivalents per g of dried extract (mg CE (g d.e.)$^{-1}$).

Ascorbic acid content

Ascorbic acid content of the crude extracts was determined according to Papoutsis *et al.* (2016c). Briefly, 3 mL of reagent solution (0.6 M H_2SO_4, 28 mM Na_3PO_4 and 4 mM ammonium molybdate) were mixed with 0.3 mL of diluted sample and incubated at 95 °C for 90 min. After incubation, the absorbance was measured at 695 nm using a spectrophotometer (Varian Australia Pty. Ltd., Vic., Melbourne, Australia). Ascorbic acid was used as standard and the results were expressed as mg of ascorbic acid equivalents per g of dried extract (mg AAE (g d.e.)$^{-1}$).

Cupric Reducing Antioxidant Capacity (CUPRAC)

CUPRAC was determined according to Papoutsis *et al.* (2016b). Briefly, 1 mL of $CuCl_2$ (10 mM) was mixed with 1 mL of neocuproine (7.5 mM) and 1 mL of NH_4Ac (pH 7.0). Subsequently, 1.1 mL of sample was added to this mixture. The mixture was incubated at ambient temperature for 1.5 h. The absorbance was measured at 450 nm using a spectrophotometer (Varian Australia Pty. Ltd., Vic., Melbourne, Australia). The results were expressed as mg of Trolox equivalents per g of dried extract (mg TE (g d.e.)$^{-1}$).

High-Performance Liquid Chromatography (HPLC)

High-performance liquid chromatography (HPLC) (Shimadzu LC-20AD, Rydalmere, NSW, Australia) was employed for the identification of individual phenolic compounds of lemon by-product crude extracts. The standards and samples were filtered through a 0.45 µm nylon filter, and 30 µL was individually injected into a C_{18} reversed-phase column (Gemini 110A 5 µm, 150 × 4.6 mm Phenomenex Australia Pty., Ltd., Lane Cove, NSW, Australia) supplied with a guard column (Gemini C_{18}, 4 × 3.0 mm). The column temperature was maintained at 30 °C (Phenomenex Therma Sphere TS-130 Column Heater, Phenomenex Australia Pty., Ltd., Lane Cove, NSW, Australia). UV detector was used for the identification of phenolic compounds. The mobile phase contained water: acetonitrile: formic acid, 95:4:1 (v:v:v) (Mobile Phase A) and acetonitrile (Mobile Phase B). The flow rate of the solvents was 1 mL per min, and the following gradient solution was used: 0 min 5% B; 15 min, 20% B; 35 min, 100% B; 40 min, 5% B; 50 min, 5% B. The analysis was stopped after 60 min. The system was equilibrated between runs for 10 min using 50% of mobile phase B. The identification of individual phenolic compounds was performed at 280 nm by comparing the retention times of the peaks obtained from the extracts to those obtained from the standard compounds. The quantification of the identified phenolic compounds was performed by an external standard method using standard curves and the results were expressed as mg g^{-1}. The standard curve for each phenolic compound (hesperidin, neohesperidin, rutin, *p*-coumaric acid, caffeic acid and sinapic acid) was linear (R^2= 0.9997, 0.9999, 0.9994, 0.9986, 0.9947 and 0.9988, respectively) in the range of 6.25-200 µg mL^{-1} methanol.

In vitro* antifungal activity determination against *Alternaria alternata

Per cent growth inhibition

The effects of two lemon by-product extracts at different concentrations (14, 7, 3.5, and 1 mg mL^{-1}) on the mycelial growth of *A. alternata* (DAR No. 83294) isolated from infected sweet cherries (*Prunus avium*), were determined according to Tao *et al.* (2014) with some modifications. Briefly, 40 ml of sterilized ¼PDA medium was mixed with the appropriate amount of lemon by-product crude extract and then poured into petri dishes (9 cm). Unamended media were used as a negative control, while fludioxonil fungicide (Scholar®) at a concentration of 2.6 µL per mL was used as a positive control. For exploring potential compounds with antifungal activity, standard compounds (hesperidin, rutin, *p*-coumaric acid and ascorbic acid) which have been identified in lemon by-product aqueous extracts were incorporated into the ¼PDA in a concentration of 5 mM (Ortuño *et al.*, 2006). Petri dishes were inoculated with a 6-day-old *A. alternata* (0.40 cm), with the mycelial surface facing up and incubated at 25 °C for 7 days. Each experiment was conducted three times and each treatment had three replicates. The diameter growth was measured by a ruler and calculated by Equation 1, while the per cent growth inhibition rate to be calculated using Equation 2 (Tao *et al.*, 2014).

$$Diameter\ growth = Xd - Xi \quad \text{(Eq. 1)}$$

Where Xd is the growth in cm after 7 days and Xi is 0.40 cm.

$$Per\ cent\ growth\ inhibition\ rate = \left[\frac{Dc-Dt}{Dc}\right] * 100\% \quad \text{(Eq. 2)}$$

Where Dc= (Diameter of control after 7 days (cm) - initial diameter (0.40) in cm) and Dt= (Diameter of treatments after 7 days (cm) - initial diameter (0.40) in cm).

Spore germination

The spore germination was determined according to Yang *et al.* (2016) with some modifications. Standard compounds (*p*-coumaric acid and ascorbic acid) at a concentration of 5 mM and crude extracts of untreated and microwave treated lemon by-

product at a concentration of 14 and 7 mg ml^{-1} were incorporated into the media (¼PDA) and poured onto a 7.5 cm sterile glass slide. Unamended media were used as a control. After solidifying, each slide was inoculated with 20 µL of fungal spore suspension (5 × 10^5 cells per mL) and placed on a 9 cm Petri dish with wet filter paper at the bottom. After 6 h incubation at 25 °C, 60 spores per slide were examined using a microscope (Olympus Bx41) and the number of germinated spores with germ tubes longer than twice the diameter of corresponding spores was counted. Each experiment was conducted three times and each treatment had three replicates.

Scanning electron microscopy (SEM)

A six-day-old fungus was subcultured onto ¼PDA dishes contained the extract derived either from the treated or untreated lemon by-product at a concentration of 14 mg ml^{-1}. Unamended media were used as a control. After incubation at 25 °C for 3 days, segments (5 x 10 mm) were cut at the periphery of the colony and were promptly placed in vials containing 2.5% glutaraldehyde in 0.05M phosphate buffer saline. The samples were kept in the solution for 48 h and then they were washed with distilled water 3 times for 10 min each time. Subsequently, the samples were dehydrated in ethanol series (30%, 50%, 70%, and 95%) for 20 min in each ethanol dilution and finally in absolute ethanol for 30 min. The samples were frozen using liquid nitrogen and freeze-dried. The dried samples were coated with gold (80 sec, 15 mA) and were observed using ZEISS SIGMA VP scanning electron microscope (SEM).

Statistical analysis

All the data have been expressed as mean ± standard deviation. All the phytochemical assays were conducted in triplicate, with an exception of the determination of individual

phenolic compounds, where five replications were used. The means of all phytochemical assays were compared using the *t*-test at $P<0.05$. The effects of each treatment on per cent growth inhibition rate, diameter growth, and spore germination were assessed by Duncan's post hoc multiple comparison test with the SPSS statistical software (version 23, IBM, Crop., NY, USA) at $P<0.05$.

Results and discussion

Polyphenol content and antioxidant capacity of lemon by-product aqueous extracts

The TPC, TF, and cupric reducing antioxidant capacity of the extracts obtained from the microwave treated by-product were 53, 13, and 49%, respectively, higher than those obtained from the untreated one ($P<0.05$) (Table 1). Moreover, the aqueous extracts of the microwave treated lemon by-product had higher ascorbic acid content (202.93±14.64 mg AAE (g d.e.)$^{-1}$) than those obtained from the untreated ones (112.93±19.23 mg AAE (g d.e.)$^{-1}$), indicating that microwave treatment of the freeze-dried lemon by-product enhances the recovery of ascorbic acid. This is in agreement with previous findings reporting higher retention of ascorbic acid in vegetables treated by heat (Cross & Fung, 1982). Moreover, previous studies have mentioned that the polyphenol content and antioxidant capacity of citrus extracts can be enhanced by the application of microwave irradiation to the dried material (Hayat *et al.*, 2010, Papoutsis *et al.*, 2016a). Three phenolic acids (caffeic acid, *p*-coumaric acid, and sinapic acid) and three flavonoids (neohesperidin, hesperidin, and rutin) were identified and quantified by HPLC in both treated and untreated lemon by-products. The contents of all individual phenolic compounds were significantly higher in the extracts obtained after lemon by-product microwave treatment compared to those obtained from the untreated by-products ($P<0.05$) (Table 2). The higher caffeic acid, *p*-coumaric acid, sinapic acid, neohesperidin,

hesperidin, and rutin yields obtained from the microwave treated by-product could be attributed to the liberation of the bound phenolics due to the heating effect during microwave treatment (Hayat *et al.*, 2010).

Effect of lemon by-product aqueous crude extracts on *A. alternata* mycelial growth

The effect of two lemon by-product extracts at different concentrations (14, 7, 3.5 and 1 mg mL^{-1}) on *A. alternata* mycelial growth can be seen in Fig. 1. Both extracts inhibited the mycelial growth of *A. alternata* in a concentration-dependent manner. The diameter growth of the fungus significantly decreased as the concentration of both extracts increased from 1 to 14 mg mL^{-1} (Fig. 1a), with the extracts obtained from the microwave treated lemon by-product showing higher per cent growth inhibition rate (ranging from 31 to 73%) than those obtained from the untreated lemon by-product (ranging from 30 to 68%) ($P<0.05$) (Fig. 1b). The diameter growth of *A. alternata* in the negative control (unamended) was 6.85 cm after 7 days storage at 25 °C, while the fludioxonil fungicide (positive control) totally inhibited the growth of the fungus. Both extracts at the concentrations of 14 and 7 mg mL^{-1} significantly altered the morphology of *A. alternata* (Fig. S1). For exploring potential compounds with antifungal activity in the extracts, the effects of four standard compounds (*p*-coumaric acid, ascorbic acid, rutin hesperidin) which have been identified in lemon by-product extracts were investigated in a concentration of 5 mM (Fig. 1). Both ascorbic acid and *p*-coumaric acid exhibited strong antifungal activity against *A. alternata*, while the two flavonoids (rutin and hesperidin) had negligible effects on the growth of the fungus (Fig. S1). These results are in accordance with previous studies which found that phenolic acids inhibited the growth of *A. alternata* infected tomatoes and sweet cherries (Pane *et al.*, 2016, Wang *et al.*, 2017). Indeed the mechanisms of action of the bioactive compounds of plants are not completely

understood (da Cruz Cabral *et al.*, 2013). However, the antifungal activity of the lemon by-product aqueous extracts could be attributed to the presence of compounds contained hydroxyl (OH) groups in their molecules, such phenolic acids and ascorbic acid, since hydroxyl (OH) groups are able to form hydrogen bonds which modify a variety of intracellular factions in the fungi (da Cruz Cabral *et al.*, 2013, Yang *et al.*, 2016). This is further supported by the enhanced antifungal activity of the extracts obtained from the microwave lemon by-products which contained higher phenolic acid and ascorbic acid contents than those obtained from the untreated ones. The negligible antifungal activity of hesperidin and rutin implies that the position of the hydroxyl (OH) group in the aromatic ring of the compounds may affect their antifungal activities. Future studies investigating synergistic and antagonistic interactions of phenolics and other bioactive compounds on the inhibition of *A. alternata* growth are encouraged.

Morphological changes detected by using scanning electron microscopy (SEM)

The hyphae of *A. alternata* grown on amended ¼ PDA petri dishes (control) was regular, with a smooth surface and linearly shaped (Fig. 2a). In contrast, both extracts at the concentration of 14 mg mL^{-1} altered the morphology of the fungus hyphae, resulting in rough surfaces, cell wall collapse and loss of linearity (Fig. 2b, and c). Additionally, the formation of a layer of extruded material that covered the mycelia was observed in the hyphae treated with both lemon by-product aqueous extracts. The extracts obtained from the microwave treated lemon by-products resulted in greater hyphae damage (Fig. 2c) compared to those obtained from the untreated one (Fig. 2b). Similar results have been observed in *A. alternata* treated with essential oils (Castro *et al.*, 2017), and in *P. italicum* treated with poplar buds extracts (Yang *et al.*, 2016). In this study the morphology changes observed could be due to the interaction of some bioactive compounds contained

in the lemon by-product extracts, such as phenolic acids or ascorbic acid, with membrane enzymes, leading to the loss of integrity of the hyphae cell walls (da Cruz Cabral *et al.*, 2013). It has been reported that phenolic compounds may alter fungus cell permeability, leading to macromolecule leakage (da Cruz Cabral *et al.*, 2013). This effect may explain the formation of a layer of extruded material that covered the mycelia of the *A. alternata* hyphae treated with the lemon by-product aqueous extracts (Fig. 2b, and c).

Effect of microwave treated and untreated lemon by-product crude extracts on *A. alternata* spore germination

In the spore germination experiment, the extract concentrations of 14 and 7 mg mL^{-1} were selected since at these concentrations high per cent growth inhibition was achieved. The effect of two lemon by-product aqueous extracts at different concentrations on *A. alternata* spore germination can be seen in Fig. 3. The spore germination was lower in the media incorporated with the extracts obtained from the microwave treated lemon by-product than those obtained from the untreated one ($P<0.05$). The effects of ascorbic acid and *p*-coumaric acid on *A. alternata* spore germination were also investigated since these extracts showed strong per cent growth inhibition activity. Both ascorbic acid and *p*-coumaric acid inhibited the spore germination of the fungus (Fig. 3). These results are in accordance with Karim *et al.* (2016) who indicated that the methanol extracts of different *Cistus* L. species inhibited the spore germination of *Geotrichum citri-aurantii* and Yang *et al.* (2016) who showed that the extracts of poplar buds inhibited the germination of *Penicillium italicum*. Given that spore germination is a process that requires metabolic energy it could be hypothesized that bioactive compounds contained in lemon by-product aqueous extracts may affect the activity of enzymes implicated in the metabolic respiration of the fungus.

The results of this study indicate that the aqueous extracts of lemon by-products contain bioactive compounds which could be potentially useful as an alternative to synthetic fungicides for controlling *A. alternata*. A large-scale application of plant extracts could be achieved by their incorporation into edible films (Guerreiro *et al.*, 2016). However, the antifungal activities of the extracts might be affected by the composition of the film, as well as the methods and conditions being applied for film drying (Mayachiew *et al.*, 2010). Therefore, future studies should be conducted investigating the incorporation of lemon by-product extracts into edible films and coatings, as well as to characterize their stability.

Conclusions

To sum up, lemon by-product aqueous extracts inhibited the growth and suppressed the spore germination of *Alternaria alternata* in a concentration-dependent manner. The *in vitro* antifungal and antioxidant activities of lemon by-product aqueous extracts can be enhanced by treating the dried lemon by-product with microwave energy. The antifungal effects of lemon by-product extracts were attributed to the presence of phenolic acids and ascorbic acid into the aqueous extracts. Lemon by-product extracts significantly changed the morphology of fungus hyphae, leading to a cell wall collapse and loss of linearity. Lemon by-product extracts could be potentially useful as an alternative to synthetic fungicides for controlling *A. alternata*. Future studies should investigate the incorporation of lemon by-product extracts into edible films and coatings, as well as to characterize their stability.

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Tables

Table 1. Total phenolic content (TPC), total flavonoid content (TF), ascorbic acid content, and antioxidant capacity (CUPRAC) of treated and untreated lemon by-products. Results are expressed as mean ± standard deviation (n=3).

	Untreated by-product	Treated by-product
TPC (mg GAE (g d.e.)$^{-1}$)	40.94±1.40^{b*}	62.82±1.58^{a}
TF (mg CE (g d.e.)$^{-1}$)	10.63±0.31^{b}	12.00±0.14^{a}
Ascorbic acid (mg AAE (g d.e.)$^{-1}$)	112.93±19.23^{b}	202.93±14.64^{a}
CUPRAC (mg TE (g d.e.)$^{-1}$)	111.14±1.39^{b}	165.53±6.08^{a}

*Values followed by different letters in the same row are significantly different at $P<0.05$, according to the *t*-test.

Table 2. Phenolic acid and flavonoid content (mg g^{-1}) of untreated and treated lemon by-products. Results are expressed as mean ± standard deviation (n=5).

	Phenolic acids			Flavonoids		
	Caffeic acid	*p*-Coumaric acid	Sinapic acid	Neohesperidin	Hesperidin	Rutin
UB	0.13±0.02^{b*}	0.19±0.01^{b}	1.21±0.02^{b}	0.28±0.01^{b}	23.79±0.31^{b}	8.54±0.21^{b}
TB	0.53±0.05^{a}	0.66±0.06^{a}	2.17±0.11^{a}	1.91±0.04^{a}	27.10±0.33^{a}	12.25±0.46^{a}

*Values followed by different letters in the same column are significantly different at $P<0.05$, according to the *t*-test.
UB: Untreated by-product
TB: Treated by-product

Figures

Fig. 1. Effect of two aqueous extracts obtained from the microwave treated and untreated lemon by-product on diameter growth (a), and per cent growth inhibition (b). Bars with different letters are statistically different at $P<0.05$ using the Duncan's post hoc multiple comparison test.

Fig. 2. Scanning electron micrographs of *A. alternata* hyphae. (a) Hyphae of untreated fungus (control); (b) Hyphae of fungus grown on ¼ PDA incorporated with extract (14 mg ml^{-1}) obtained from untreated lemon by-product; (c) Hyphae of fungus grown on ¼ PDA incorporated with extract (14 mg ml^{-1}) obtained from microwave treated lemon by-product.

Fig. 3. Effect of two aqueous extracts obtained from the microwave treated and untreated lemon by-product on *A. alternata* spore germination. Bars with different letters are statistically different at $P<0.05$ using the Duncan's post hoc multiple comparison test.

Supplementary material

Fig. S1. Hyphal morphology of *A. alternata*