Green extraction of polyphenols from citrus peel by-products and their antifungal activity against *Aspergillus flavus*

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**ABSTRACT**

*Aspergillus flavus* is a pathogenic fungus associated with food safety issues worldwide. This study investigated the antifungal activity of citrus peel extracts prepared using food-grade solvents (hot water or ethanol). Mandarin (*Citrus reticulata*) peel ethanol extracts inhibited the mycelial growth of *A. flavus* (39.60%) more effectively than those of orange (32.31%) and lemon (13.51%) after 7 days of incubation. The growth of *A. flavus* could be completely inhibited by mandarin extracts at 300–400 mg mL⁻¹, depending on the extraction solvent. Solid-phase extraction (SPE) separated the polyphenol-rich fractions, which showed up to 40% higher antifungal activity than crude extracts. Twelve polyphenols (2 phenolic acids and 10 flavonoids) were identified by HPLC-MS and some of these had antifungal activity against *A. flavus*. In conclusion, citrus peels are promising bioresources of antifungal agents with potential applications in food and other industries.

1. **Introduction**

Mycotoxigenic fungi are considered as major threats to food safety worldwide. The fungi cause food spoilage leading to loss and waste, but can additionally produce toxic mycotoxins that pose serious health problems to both human and livestock (Jing et al., 2014). Aflatoxins are mycotoxins produced by *Aspergillus flavus* which are of particular concern because of their hepatotoxicity and carcinogenicity (Abdel-Kareem, Rasmey, & Zohri, 2019). A good approach to prevent aflatoxins in food is by inactivating *A. flavus* and preventing its growth (Liu, Galani, & Orfila, 2020). The usage of biological metabolites to prevent fungal growth is regarded as a safe, effective and environmentally friendly preventative method (Rasheed et al., 2020).

Citrus (genus *Citrus* L.) is one of the most important fruit crops, growing widely in tropical and subtropical regions. According to the FAO, >140 million tons of citrus were produced in 2019 (FAOSTAT, 2021). Apart from fresh produce, citrus fruits are processed into juice, canned or dehydrated products, marmalades, jams, and flavouring agents. Around 50–60% of the fruit weight, including peels, seeds and segment membranes, are generated as by-products after processing (Mahato, Sinha, Sharma, Koteswararao, & Cho, 2019). These citrus by-products contain bioactive compounds such as vitamins, minerals, phenolic compounds, terpenoids and dietary fibre (Mahato et al., 2019). Polyphenols are bioactive molecules widely found in plant species, affecting their morphology, growth, reproduction and resistance to pathogens and environmental stresses (Bahorun, Luximon-Ramma, Crotier, & Aruoma, 2004). Flavonoids are the most common group of polyphenols in plants, playing important roles in plant responses (Xi, Fang, Zhao, Jiao, & Zhou, 2014) and also showing antifungal activities (Al Aboody & Mickyamar, 2020). The most abundant flavonoids in citrus have been identified as naringin, hesperidin, narirutin, and neohesperidin (Xu, Liu, Chen, Ye, & Shi, 2009).

Studies have shown antibacterial activity of citrus metabolites towards pathogenic gram positive and gram negative bacteria that cause human diseases (Lemes et al., 2018), animal diseases (El-Desoukey, Saleh, & Alhowamil, 2018), and food spoilage (Ben Hsouna, Ben Halima, Smaoui, & Hamdi, 2017). Regarding antifungal properties, distilled citrus essential oils (mainly containing terpenes) have shown to inhibit *Aspergillus* sp., *Penicillium* sp, *Fusarium* sp., *Candida* sp., *Cladosporium* sp., *Eurotium* sp., and *Rhizopus* sp. (Jing et al., 2014). Studies on the...
antifungal activity of citrus polyphenols are limited. One study showed that purified flavanones (naringin at concentration of 68 mg mL\(^{-1}\), hesperidin at 153 mg mL\(^{-1}\) and neohesperidin at 153 mg mL\(^{-1}\)) could inhibit growth of \(A.\ flavus\) by 33–41% (Salas, Cézil, Geronazzo, Daz, & Resnik, 2011). These polyphenols are proposed to inhibit the fungus by altering the ultrastructure of the fungal cell walls and endomembrane system (Pok et al., 2020). In addition, these three flavanones were found to reduce the accumulation of aflatoxin \(B_1\) produced by \(Aspergillus\ sp.\) by 80% to 100% (Pok et al., 2020). However, there is limited knowledge on the activity of food-grade extracts, which may contain mixtures of polyphenols, as well as other bioactives. Most studies that investigated extraction of polyphenols from plant materials used organic solvents (Karim et al., 2016; Molotti et al., 2020; Olatunji, Joy, & Irene, 2019). In the food industry, the safety and sustainability of these solvents, as well as policy restrictions, calls for green extraction solvents to be considered. Therefore, this study aimed to investigate the antifungal properties of citrus peel extracts, comparing the efficacy of water and ethanol as food-grade extraction solvents. Solid phase extraction (SPE) was then applied to fractionate the crude extracts. The extracts were analysed for total polyphenol, total flavonoid content, as well for polyphenol composition by HPLC. The hypothesis is that citrus fruit peel extracts prepared with food-grade solvents show antifungal bioactivity and can be considered as sustainable antifungal agents for a range of industry applications.

2. Material and methods

2.1. Plant material and chemicals

Orange (\(Citrus\ sinensis\)) and lemon (\(C.\ limon\)) peels were obtained from Biopower Technologies Limited (BioPower, UK). Mandarin (\(C.\ reticulata\)) peel was obtained from Xiangshan Huayu Foodstuffs Co. Ltd (China). The peels were diced, dried at 65 \(^\circ\)C and then micronized to particle size of < 150 \(\mu\)m. All the samples were packed in polythene bags and stored at ~20 \(^\circ\)C until needed.

Amberlite (R) XAD-7HP resin (20–60 mesh), Folin-Ciocalteu, \(Na_2CO_3\), gallic acid, and sodium acetate were purchased from Sigma (UK). Ethanol was procured from VWR (USA). \(AlCl_3\) was purchased from Honeywell (USA). \(tert\)-Butylhydroquinone (TBHQ) was from Aldrich. Dimethyl sulfoxide (DMSO) was from Fluorochem (UK). Milli-Q water was used for extraction. Reagents used in HPLC analysis were of analytical grade. Formic acid, acetonitrile was purchased from Merck Life (UK). HPLC grade standards (purity > 97%) were used. Naringenin narirutin, naringin, hesperetin, hesperidin, neohesperidin, quercetin, rutin, isorhoifolin, rhoifolin, luteolin, cymaroside, didymy, poncirin, eriodictyol, eriocitrin, neoeiocitrin, isorhamnetin, diosmetin, neodiosmin, sinensetin, tangeretin, taxifolin, nobiletin, and protocatechuic acid were purchased from Extrasynthese (France). Quercetin, \(p\)-coumaric acid, chlorogenic acid, ferulic acid and gallic acid were obtained from Sigma (UK). Apigenin was purchased from PhytoLab (Germany), \(p\)-hydroxybenzoic acid from SAFC (USA), vanillic acid from Alfa Aesar (USA), and caffeic acid from Cayman Chemical (USA).

2.2. Fungal strain and culture conditions

\(A.\ flavus\) 9643 (non-toxigenic strain that does not produce aflatoxins) was purchased from ATCC (UK). The fungus was cultivated in 24 g L\(^{-1}\)potato dextrose broth (PDB, Sigma, UK), for 2 days. Culture medium (0.5 mL) containing conidia was spread on 15 mL of 39 g L\(^{-1}\)potato dextrose agar (PDA, Sigma, UK) in 9 mm petri dish, the dish was incubated at 30 \(^\circ\)C under dark conditions for 7 days, and the diameter of the fungal colony was measured to 0.1 mm with a ruler every day.

2.3. Citrus peel extraction

Ten grams of dried citrus peel powder were extracted in 200 mL of absolute ethanol or water at 60 \(^\circ\)C in shaking water bath for 2 h, followed by centrifugation at 3220 g for 10 min. The supernatant was filtered through Whatman #1 filter paper to separate any remaining solid residue. The extraction process was repeated twice. The supernatants were finally collected and combined. For antifungal experiments, the extraction solution was concentrated by evaporation under vacuum using a Genevac (Fisher Scientific, UK) at room temperature, and then freeze-dried (Labconco, UK). The extracts were stored at ~20 \(^\circ\)C.

2.4. Solid phase extraction (SPE) of crude mandarin extracts

Mandarin peel polyphenols were fractionated by SPE. Pre-conditioned amberlite (R) XAD7HP resin (4.8 g) was added to 8 mL of 5-fold pre-concentrated extracts. The mixture was then allowed to stand for 24 h at room temperature. Later, the resin was washed 3 times with 10 mL distilled water. The unbound extract and washing solutions were collected as the washing fraction (WF). After washing, the resin was eluted 4 times with 10 mL of absolute ethanol. The elution solutions were collected and combined as the elution fraction (EF). Both WF and EF were concentrated in a rotary evaporator (Heidelberg, Germany) at 60 \(^\circ\)C, and then freeze-dried. All samples were stored at ~20 \(^\circ\)C until use.

2.5. Determination of the effect of crude and SPE citrus extracts on \(A.\ flavus\) mycelia growth

The antifungal activity of citrus extracts against \(A.\ flavus\) mycelia growth was based on agar dilution test using PDA medium (Prakash et al., 2011). Citrus extracts (150 mg) were dissolved 1 mL of 10% DMSO and added to 15 mL molten PDA at around 50 \(^\circ\)C to achieve a final concentration of 10 mg mL\(^{-1}\). PDA medium containing 1 mL of DMSO solution was used as negative control, and 10 mg mL\(^{-1}\)of tert-Butylhydroquinone (TBHQ) as positive control. To avoid microbial contamination, the PDA medium with extracts was autoclaved at 121 \(^\circ\)C for 15 min before pouring into petri dishes. Autoclaving was found not to affect antifungal activity significantly (Supplementary Data S1). Next, a 4 mm medium disc with fungi cut from 2-day-old \(A.\ flavus\) solid culture was placed at the centre of each petri dish. The dishes were placed in an incubator at 30 \(^\circ\)C for 7 days. The diameter of the fungal colony was measured every day.

The antifungal activity was calculated as:

\[
\text{Antifungal activity} (\%) = \left(1 - \frac{D_c}{D_t}\right) \times 100
\]

Where, \(D_t\) the diameter of fungal colony in the treated dishes; \(D_c\) the diameter of fungal colony in the negative control dishes.

2.6. Effect of concentration on \(A.\ flavus\) mycelia growth of crude mandarin extracts

Requisite amounts of water and ethanol mandarin extracts were dissolved in 15 mL molten PDA to get the final concentration of 5, 10, 50, 100, 200, 300, 400 mg mL\(^{-1}\) followed by autoclaving, plating, inoculation and incubation, as previously described.

2.7. Identification and quantification of polyphenols

2.7.1. Determination of total phenolic content (TPC)

TPC of extracts was determined by the Folin-Ciocalteu reagent method, based on Singleton, Orthofer, and Lamuela-Raventós (1999) with some modifications. Briefly, 10 \(\mu\)L of sample was mixed with 40 \(\mu\)L of Folin-Ciocalteu (12.5%) and 150 \(\mu\)L of \(Na_2CO_3\) solution (4%). The mixed sample was incubated for 30 min at room temperature in the dark. The absorbance was then measured at 765 nm with plate reader (Tecan, Switzerland). Ten \(\mu\)L of 50% ethanol solution was used as negative control. The same process was applied to the standard solution.
of gallic acid (7.81–500 mg mL\(^{-1}\)) and the obtained standard curve was used to calculate the TPC of samples, expressed as µg gallic acid equivalent (µg GAE).

2.7.2. Determination of total flavonoids content (TFC)

TFC of each extract was determined by aluminium chloride colorimetric assay following the method described by Sembiring, Elya, and Sauriasari (2017) with a slight modification. Briefly, 50 µL of each sample was orderly mixed with 10 µL of 10% AlCl\(_3\), 150 µL of 96% ethanol and 10 µL of 1 M sodium acetate. After mixing, the reaction solution was incubated for 45 min at room temperature in the dark. The absorbance was measured at 415 nm with plate reader. Fifty solution was incubated for 45 min at room temperature in the dark. The absorbance was measured at 415 nm with plate reader. Fifty µL of 50% ethanol solution was used as negative control. Diluted rutin (6.25–200 mg mL\(^{-1}\)) was used to make the standard curve and determine the content of flavonoids, which was expressed as µg rutin equivalents (µg RE).

2.7.3. HPLC analysis of phenolics compounds

The phenolic compounds were determined by HPLC method as previously reported by Huang et al. (2018) with some modifications. The chromatography was carried out with Agilent HPLC 1200 Series comprising an autosampler set at 4 °C, a UV detector (DAD) set at 254, 280, 330 and 540 nm, and a column oven (Agilent Co., UK). Separation of compounds was done on an Agilent Eclipse XDB-C18 4.6 × 250 mm, 5 µm reverse phase column, maintained at 40 °C. The mobile phase consisted of solvent A (0.5% formic acid) and solvent B (acetonitrile) with the flow rate of 0.5 mL/min. Gradient elution was performed as follows: 0–30 min, 10–25% B; 30–40 min, 25–70% B; 40–50 min, 70–90% B; 50–52 min, 90% B; 52–54 min, 10% B. The injection volume was 10 µL.

Thirty-four phenolic compounds were identified using their retention time at indicated wavelength (Fig. 2S).

2.8. Statistical analysis

Compound analyses and antifungal experiments were carried out in triplicate. Data were analysed using GraphPad 7 and presented as means ± standard deviation. For comparison of antifungal activity of different citrus peel extracts, a two-way ANOVA followed by a Tukey-test (p < 0.05) was used to compare the difference of two factors (treatments × incubation day). For phenolic composition analysis, one-way ANOVA with a posthoc Tukey-test (p < 0.05) was applied to compare the difference between the extraction methods.

3. Results and discussion

3.1. The effect of citrus extracts on A. flavus mycelia growth

The effect of orange, lemon and mandarin peel ethanol crude extracts at a concentration of 10 mg mL\(^{-1}\) on A. flavus mycelia growth is shown in Fig. 1. Two-way ANOVA analysis showed that there were significant effects (p < 0.0001) of both treatments (positive control and citrus peel extracts) and incubation time on the antifungal activity. Then the pairwise comparison demonstrated that three crude extracts showed significant inhibitory activity (p < 0.05) compared to control (Fig. 1a). Among the extracts (Fig. 1b), mandarin peel extract displayed the highest inhibition activity (ranging from 53 to 40% over 7 days), followed by the orange extract (40–32%). Over 7 days, all extracts showed a decrease in antifungal effect. The activity of mandarin and lemon extracts decreased by 13% on day 7 compared to day 1, while the reduction was relatively lower for orange extract (8%). Our results are similar to the report of Okwu, Awurum, and Okoronkwo (2007) who compared the inhibitory effect of five citrus peel extracts, including C. reticulata, C. aurantiifolia, C. limonum, C. sinensis and C. vitis, against F. oxysporum. Among these citrus peel extracts, C. sinensis showed the highest (83.55%) inhibitory effect compared to other extracts (42.15–71.10%). The higher activities observed by these authors suggested that F. oxysporum may be more susceptible to citrus extracts than A. flavus. This could also be due to the differences in origin and varieties of the citrus, which influence their content in antimicrobial compounds. In the present study, the highest antifungal activity was observed for mandarin peel extract, and therefore mandarin was selected for further work.
Fig. 2. Effect of concentrations (mg/mL) of water (a) and ethanol (b) extracts on *A. flavus* colony growth at 25 °C during 7 days.

Fig. 3. Antifungal activity (%) of a) water and b) ethanol mandarin peel extracts and their SPE fractions (10 mg/mL) at 25 °C during 7 days.
The total phenolic and total flavonoid content in mandarin peel crude and SPE extracts.

| Solvent | Crude Extracts | Washing Fractions | Elution Fractions |
|---------|----------------|-------------------|------------------|
|         | Yield (%)      | Recovery (%)      | Recovery (%)     | Recovery (%)     |
| Water   | 42.24          | 6242.80 ± 198.56  | 2822.00 ± 45.2   | 3253.87 ± 52.12 |
| Alcohol | 31.88          | 4244.80 ± 222.33  | 103.52 ± 41.25   | 153.60          |

Phenolic Content: Water 28.22 ± 45.2, Alcohol 103.52 ± 41.25.
Flavonoid Content: Water 243.17 ± 7.72, Alcohol 183.04 ± 8.80.

Data are presented as means ± SD of triplicate samples. * represents significant differences compare ethanol crude extract to water crude extract. ***, p < 0.0001; **, p < 0.001; *, p < 0.05; ns, p > 0.05.

**a)** GAE, gallic acid equivalent; RE, rutin equivalent.

**b)** Recovery (%), the percentage of each fraction recovered from crude extracts.

### 3.2. Dose-dependent inhibitory activity of crude mandarin extracts on mycelia growth of A. flavus

To evaluate the effect of extract concentrations on fungal mycelia growth, water and ethanol crude extracts were added to the PDA medium to a final concentration of 1, 5, 10, 50, 100, 200, 300 and 400 mg mL\(^{-1}\). The results show that the fungal inhibition increased with the concentration of extracts (Fig. 2). A. flavus was 100% inhibited in first two days with 200 mg mL\(^{-1}\)of ethanol extract, while antifungal activity decreased about 15% after 5 days. The minimum inhibitory concentration (MIC) of A. flavus for water and ethanol mandarin extracts over 7 days were 400 and 300 mg/mL respectively. The difference might be due to the variation in the composition of samples prepared with different extraction solvents. The preliminary research of the non-aflatoxigenic strain and aflatoxigenic strain (Penicillium sp. at concentrations ranging 50 to 100 µg mL\(^{-1}\)) promoted fungal growth by 4.61–19.74% after 7 days. Similarly, the EF of ethanol extract inhibited A. flavus growth by 65.13–70.47%, and WF increased fungal growth up to 7.15%. Therefore, SPE technique significantly (p < 0.0001) increased antifungal ability for both water extract (by about 30% compared to the crude) and ethanol extract (by about 20%), presumably by concentrating antifungal components in the EF and removing the ingredients that did not help fungal inactivation (or actually promoted fungal growth) into the WF. In particular, sugars, which could promote fungal growth, were washed into the WF (data not shown).

### 3.4. Identification and quantification of polyphenols in mandarin peel extracts

#### 3.4.1. Total phenolic/flavonoid content of crude and SPE extracts

The content of total polyphenols and flavonoids was significantly different between water and ethanol crude extracts (p < 0.05) (Table 1). The results show that water could extract more phenolic compounds than absolute ethanol at 60 °C. In contrast, in the study by Lapornik, Prosek, and Golic Wondra (2005), TPC extracted by organic solvents (70% methanol and ethanol) was higher compared to water extracts. The TPC of crude water and ethanol extracts were 6242.80 and 4244.80 µg GAE, while the TFC were 3149.78 and 2080.72 µg RE respectively. The TPC and TFC measured in the present study were lower compared to those of methanol extracts of Citrus reticulata fruits peel (Zhang et al., 2014).

The moderate-polar resin amelinite (R) XAD7HP used in this study has been previously utilized for polyphenol purification from plant crude extracts, such as olive leaf (Karakaya, 2011) and blackcurrant (Rose et al., 2018). The yield (dry weight as a proportion of crude extract) of the eluting (EF) and washing fractions (WF) after SPE of mandarin extracts are presented in Table 1. The TFC of water and ethanol in EF were 2470.22 and 1749.42 µg GAE, which was about 78.43% or 84.08% of what is found in crude extracts respectively. However, significant amounts of phenolic compounds were washed into the WF, which were 2822.00 µg GAE for water extracts and 1752.53 µg GAE for ethanol extracts.

The Folin-Ciocalteu method is one of the most extensively used methods for the quantification of phenolic compounds content. However, TPC assay has some limitations. On the one hand, some compounds (e.g. sugars) are able to react with Folin-Ciocalteu reagent (Farooque, Rose, Benhoud, Blackburn, & Rayner, 2018). On the other hand, methoxylated polyphenols do not react with the reagent (Maggraf, Karnopp, Rosso, & Granato, 2015). Thus, TPC would overestimate the ‘phenolic content’ when the sample contains high content of sugars, while would underestimate the total phenolic content when the sample...
Fig. 4. HPLC chromatogram of mandarin peel extracts at (a) 254 nm, (b) 280 nm and (c) 330 nm. WE, water extract; EE, ethanol extracts; CE, crude extracts; WF, washing fraction; EF, elution fraction. Peaks: 2, Protocatechuic acid (internal standard); 7, ρ-coumaric acid; 8, Eriocitrin; 9, Rutin; 12, Ferulic acid; 13, Taxifolin; 14, Narirutin; 19, Hesperidin; 22, Didymin; 24, Eriodictyol; 32, Sinensetin; 33, Nobiletin; 34, Tangeretin.
has methoxylated polyphenols. Similarly, in TFC reaction, there are two
types of complexes formed: acid stable complexes with flavones and
flavonols having a C-4 carbonyl group and a C-3/5 hydroxyl group, and
acid unstable complexes with catechol hydroxyl groups in the A-ring or
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their hydrophobicity (Chen et al., 2020). Therefore, aqueous and organic solvent in this study showed similar behaviour for phenolic extraction. When it comes to individual compounds, the difference in water and ethanol extracts could be explained by their Log\textsubscript{10} P values. Hence, amongst the 12 identified polyphenols, \textit{p}-coumaric acid (1.43), sinensetin (3.19), nobiletin (3.37) and tangeretin (3.78) have a relatively higher theoretical Log\textsubscript{10} P value, which correlated with significantly higher (p < 0.05) solubility in ethanol than water. On the contrary, the Log\textsubscript{10} P of hesperidin and eriocitrin was −0.55 and −1.06, so that significantly more of these compounds were found in water extract (1643.60 and 87.24 \(\mu\)g/g respectively) than ethanol extract (1346.44 and 82.60 \(\mu\)g/g respectively). The rest of the 6 phenolics were extracted by two extraction solutions in similar quantities (p > 0.05), and this corresponded to their Log\textsubscript{10} P values which are closer to zero.
compared to above compounds.

XAD-7HP resin is an aliphatic non-ionic acrylic ester polymer with moderate polarity, showing both hydrophobic and hydrophilic behaviours, thus it is a good choice for isolating the components with both characteristics (Farooque et al., 2018; Karakaya, 2011). Polar solvent (water) was used in washing steps to remove polar molecules (e.g. sugars), while nonpolar solvent (ethanol) disrupts the interaction between hydrophobic components and the resin that elutes the adsorbate.

According to the theoretical LogP of narirutin and hesperidin, they have stronger hydrophilic characteristic than other phenolics, so a small amount of them were eluted during washing steps but not others. From present results, XAD-7HP showed a ‘good’ isolation ability of phenolics from crude extracts, as the recovery of each component was between 80 and 120% (hesperidin in water extract was 79.38%, eriocitrin and rutin in ethanol extract was 78.43% and 79.75% respectively). The lost phenolics might be retained in the resin. To improve the elution efficiency, SPE steps could be optimized with gradient elution solutions (Karakaya, 2011).

Compared to Folin-Ciocalteu and AlCl₃ method, HPLC analysis more completely and accurately reflected the phenolic compounds in extracts. However, the Folin-Ciocalteu and AlCl₃ assays are still among the most common and easy-operated methods for comparing TPC and TFC between samples, and allow monitoring the distribution of phenolic compounds during extraction and SPE processing. Therefore, these two methods still could be applied as quick assays for total phenolic and flavonoid detection in research and industry.

3.4.3. Proposed mechanism of mandarin polyphenols for their fungal inhibition effect

The antifungal properties of the polyphenols maybe attributed to their functionality such as molecule size and functional groups (e.g. number and position of hydroxyl groups, their substitutions, with/without glycosylation and its position (Makarewicz, Drozdz, Tarko, & Duda-Chodak, 2021; Sanver, Murray, Sadeghpour, Rappolt, & Nelson, 2016). For example, the aglycone hesperitin displayed a higher antifungal activity than the glycoside hesperidin. Meanwhile, neohesperidin has a higher activity than hesperidin, suggesting the configuration of the glycoside also influences activity. Furthermore, polyphenols can be chemically modified. For example, Salas et al. (2011) showed that flavonoids esterified with butyrate and decanoate had higher fungal inhibition ability than unsubstituted molecules, while esterification with stearate impeded the antifungal ability.

Multiple mechanisms underpinning antifungal actions of polyphenolic compounds have been suggested including: 1) inhibition of glucans and chitin biosynthesis resulting in deformation of fungal cell wall, 2) disruption of plasma membrane and its biosynthesis leading to leakage of intracellular components, 3) suppression of fungal nucleic acid metabolism through inhibition of mitochondrial processes, 4) inhibition of metabolic enzymes (Al Aboody & Mickymaray, 2020; Makarewicz et al., 2021). The extract produced in the present study contained a mixture of polyphenols which may act through above through a combination of effects.

In the mandarin extracts, narirutin and hesperidin were the most abundant polyphenols. It was demonstrated that the C2 – C3 double bond in heterocyclic C ring, rendering a planar structure, has stronger interaction with biological membrane components than nonplanar chemical structure, while the attached glycoside groups sterically hinder the interaction (Salas et al., 2011; Sanver et al., 2016). Both narirutin (Fig. 5f) and hesperidin (Fig. 5g) lack of C2 – C3 double bond and have a glycoside group, they may have weaker interaction with cell membrane than rutin (third abundant compound, Fig. 5c). In addition, the aromatic and planar structure of the C ring with a −C＝O group at position of C4 in C ring allows the formation of a pseudo ring with a −OH group at position of C5 in A ring, leading to the disruption of enzyme binding and activity (Makarewicz et al., 2021). This kind of configuration is present in eriocitrin (Fig. 5b), rutin (Fig. 5c), taxifolin (Fig. 5e), narirutin (Fig. 5j) and eriodictyol (Fig. 5i). Therefore, the most abundant compounds in mandarin extracts may not be the most active, but other less abundant compounds may play the more the important role in antifungal properties. We can also not rule out synergistic effects between the compounds (Pok et al., 2020).

4. Conclusion

The present study provided evidence that citrus peel extracts inhibit the growth of A. flavus, and therefore are good candidates for further investigation as antifungal ingredients. Amongst the citrus extracts investigated, mandarin was the most effective. The antifungal activity of water extracts was less effective than ethanol extracts but still showed considerable activity. The MIC of both extracts was higher than other studies on plant extracts inhibiting A. flavus. The MFC was not determined in this study. SPE was used to fractionate antifungal components from crude extracts and significantly improved antifungal capability. Ten flavonoids and two phenolic acids were identified and quantified in the extracts. Among these phenolic compounds, narirutin and hesperidin was the most dominant in both extracts.

This study provides novel information on antifungal bioactivity of citrus fruit peel extracts prepared with food-grade solvents and potential usage of citrus peel by-products to improve food safety and extend shelf-life. These extracts could developed as antifungal agents for food raw material storage (e.g. cereals, spices), or considered as natural antifungal ingredients added directly to food or packaging material. For practical applications, the experiments need to be scaled up and wider considerations taken (e.g. toxicity, sensory aspects). Future work should elucidate mechanisms of action, and the effect of these extracts on aflatoxin production.

Declarations of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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