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Approaches to Reduce *Aspergillus flavus* and Aflatoxin Contamination through Utilization of Agricultural By-Products

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Submitted in accordance with the requirements for the degree of Doctor of Philosophy

University of Leeds

School of Food Science & Nutrition

March 2022

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The candidate confirm that the work submitted is her own, except where work which has formed part of jointly authored publications has been included. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others. Details of the jointly authored publications and the contributions of each author are outlined on the next page.

The following published material has been included in the thesis.

1. Liu, Y., Galani Yamdeu, J.H., Gong, Y. Y. and Orfila, C. 2020. A review of postharvest approaches to reduce fungal and mycotoxin contamination of foods. *Comprehensive Reviews in Food Science and Food Safety*. pp.1-40. (Chapter 2)

YL (the candidate) performed the systematic literature review and critical evaluation of literature data. YL drafted the manuscript. All authors contributed ideas to the design and organization of the work. All authors edited and reviewed the manuscript.

2. Liu, Y., Benohoud, M., Galani Yamdeu, J. H., Gong, Y. Y. and Orfila, C. 2022. Green extraction of polyphenols from of citrus peel by-products and their anti-fungal activity against *Aspergillus flavus*. *Food Chemistry X*. **12**(30), 1-10. (Chapter 3)

YL (the candidate) designed and performed the experiments and processed experimental data. YL drafted the manuscript. All authors contributed ideas to the design and organization of the work. All authors edited and reviewed the manuscript.

3. Liu, Y., Xia, L., Galani Yamdeu, J. H., Gong, Y. Y. and Orfila, C. Removal of aflatoxin B_1 in aqueous solution by adsorption to corn by-products. To be submitted to *Bioresource Technology*. (Chapter 4)

YL (the candidate) designed and performed the experiments and processed experimental data. XL developed the detection method. YL drafted the manuscript. All authors contributed ideas to the design and organization of the work. All authors edited and reviewed the manuscript.

Conference Poster Presentation

Liu, Y., Gong, Y. Y. and Orfila, C. Mitigation against aflatoxin B_1 contamination by binding to plant cell wall polysaccharides. XV Cell Wall Meeting, Cambridge, UK, 7-12 July 2019

Abstract

The contamination of agricultural and food products by mycotoxin-producing fungi causes major food loss worldwide. Besides, mycotoxins have been shown to cause a number of human diseases. Current research to develop approaches to prevent contamination of food by mycotoxin-producing fungi is a priority to improve food safety and reduce food loss. The aim of this thesis was to develop new approaches to reduce aflatoxin contamination through utilisation of agricultural by-products as anti-fungal agent or mycotoxin adsorbent.

In chapter 2, a comprehensive review of the literature was undertaken. Decontamination approaches could be classified as physical, chemical or biological methods, and evaluated for their effectiveness at preventing fungal growth, reduce mycotoxin production or remove mycotoxins from contaminated foods. Main findings were that multiple approaches are needed along the food chain, as there is not one 100% effective approach. Biological approaches such as using plant-derived compounds as anti-fungal agents seem effective and safe, and were investigated in the experimental chapters.

In previous studies, citrus extracts have been demonstrated to have antimicrobial effects. Therefore, the aim of chapter 3 was to evaluate the inhibition potential of citrus peel extracts on *Aspergillus flavus* (*A. flavus*) growth. Orange, lemon and mandarin peels were extracted using water or ethanol. Mandarin peel ethanol extracts showed the highest activity against myceliar growth of *A. flavus* (39.60%) compared to those from orange (32.31%) and lemon (13.51%) peel during a 7-day incubation. The growth of *A. flavus* could be completely inhibited by mandarin extracts at 300 mg/mL for ethanol extract and 400 mg/mL for water extract. Solid phase extraction (SPE) was applied to separate the polyphenol-rich fractions, which raised the antifungal ability by 40% compared to crude extracts. Composition analysis by high performance liquid chromatography (HPLC) identified, 12 polyphenols in mandarin peel extracts, narirutin and hesperidin were the most abundant.

In previous studies, native or modified corn by-products have been shown to remove carcinogens and heavy metals from water and liquid foods. The aim of chapter 4 was to investigate the

potential of corn by-products to remove aflatoxin B_1 (AFB_1) from aqueous matrix. Corn cob powder (CCP) could adsorb up to 98% of AFB_1 in 1 h. The adsorption was significantly affected by powder:AFB_1 ratio, particle size and adsorption duration, but less affected adsorption temperature and pH. Interestingly, individual cell wall components of corn cob (cellulose, arabinoxylan, xylan, lignin) did not display a significant effect on adsorption. Theoretical models suggest a pseudo-second-order adsorption mechanism, primarily driven by contact surface interactions. Thus, the AFB_1 adsorption may be attributed to the microstructure of CCP.

In conclusion, this project demonstrated the utilization of agro-industrial by-products on both antifungal activity and aflatoxin adsorption at laboratory level. Biological approaches to reduce fungal growth and aflatoxin contamination seem promising to reduce food loss and prevent human disease. Further research is needed to ascertain their safety and cost-effectiveness.

Acknowledgements

Throughout doing my PhD project I have received a great deal of support and assistance. First, I would like to thank my esteemed supervisors, Prof. Caroline Orfila and Prof. Yun Yun Gong for their invaluable guidance, continuous support and infinite patience. Their immense knowledge and plentiful experience open my eyes, guide my study and inspire me to keep on improving.

I also appreciate all the support I received from my parents, Yong Qian Liu and Gui Lin Yi, and my boyfriend, Ze Chuan Zhao. None of this work would be possible without their love, patience, concern, understanding and encouragement in the past few years, especially the tough time during COVID-19 pandemic.

Besides, I would like to give a sincere thanks to Dr. Joseph Hubert Galani Yamdeu and Dr. Meryem Benohoud for their kindness help on experimental progress and papers writing, which benefits me a lot. I would also like to thank Miles, Sara, Jo, Neil and Ian for their helpful technical support during my studies.

Finally, I would like to thank my dear friends, lab mates, and colleagues from School of Food Science and Nutrition – Lei, Dorothy, Zi Xuan, Lia, Ze Xi, Holly, Jin, Can Can, Catherine, Lily, Ndashe, Scarlet, Suvro, Ukpai, Ya, Yu, Hang, Vicente, Damien, Yun Qing, Sumali, Rui Xian, Shu Ning, Ming Duo, Chrysa, Alex, Yoga, Sam, Lu, Denise and Marjorie, for their unselfish help and courage, as well as a cherished time spent together in Leeds.

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List of Abbreviations

ACSH	Amorphous calcium silicate hydrate
A. flavus	*Aspergillus flavus*
AFB_1	Aflatoxin B_1
AFB_2	Aflatoxin B_2
AFG_1	Aflatoxin G_1
AFG_2	Aflatoxin G_2
AFM_1	Aflatoxin M_1
AFP_1	Aflatoxin P_1
AFPs	Antifungal peptides
AFQ_1	Aflatoxin Q_1
AFs	Aflatoxins
AMPs	Antimicrobial peptides
a_w	Water activity
BCAs	Biocontrol agents
CBP	Corn bran powder
CCP	Corn cob powder
CCSH	Crystalline calcium silicate hydrate
CE	Crude extract
CEC	Cation exchange capacity
CFU	Colony forming unit
Chit A	Chitinase A

Chit B	Chitinase B
CYP_{450}	Cytochrome P_{450} enzymes
DON	Deoxynivalenol
EDTA	Ethylene diamine tetraacetic acid
EF	Elution fraction
EO	Essential oil
EOW	Electrolyzed oxidizing water
FAO	Food and Agriculture Organization
FB_1	Fumonisin B_1
FB_2	Fumonisin B_2
FBs	Fumonisins
FLW	Food loss and waste
HBV	Hepatitis B
HCC	Hepatocellular carcinoma
HPLC	High performance liquid chromatography
HSCASs	Hydrated sodium calcium aluminosilicates
LOD	Limit of detection
LOQ	Limit of detection
MA	Modified atmosphere
MAP	Modified atmosphere package
MC	Moisture content
MFC	Minimum fungicidal concentration
MIC	Minimum inhibitory concentration
MLs	Maximum limits
MW	Molecular weight

NaDCC	Sodium dichloroisocyanurate
NEW	Neutral electrolyzed water
NIV	Nivalenol
Orts	Organo-rectorites
OTA	Ochratoxin A
PAT	Patulin
PDB	Potato dextrose broth
PDA	Potato dextrose agar
PMF	Polymethoxylated flavone
RH	Relative humidity
RT-PCR	Reverse transcription polymerase chain reaction
SPE	Solid phase extraction
TFC	Total flavonoids content
TPC	Total phenolic content
UVC	Ultraviolet C
WHO	World Health Organization
WF	Washing fraction
ZEN	Zearalenone

Chapter 1: General Introduction

1.1 Fungal infection and mycotoxin contamination of food crops

Fungi are a part of the natural environment; thus they play very important roles in the food system. They have important applications in food production as food sources or microbial fermentation aids, but can also cause of fungal infection on foods (Benedict et al., 2016). Fungal infection accounts for 70 to 80% of food losses caused by microbial contamination (Moore et al., 2011). Every year, one third of all crops are destroyed by fungi, which directly cause losses to five of the most important crops (rice, wheat, maize, potatoes, and soybean). If these losses can be avoided, 595 million of population will be fed (in 2011) (Fisher et al., 2012; Almeida et al., 2019). In addition, mycotoxins produced by some fungi have a huge impact on food security and economic value of crops as well. There are about 25% of the global food and feed contaminated by mycotoxins (Eskola et al., 2019). Apart from the health losses from mycotoxin consumption, the crops may be rejected by customs or excluded from high-value markets due to high mycotoxin contamination, which causes income reduction to crop-producing countries (Udomkun et al., 2017).

Aspergillus sp. and their metabolites, aflatoxins, are responsible for one of the most adverse economic and health problems for farmers and consumers globally (Jallow et al., 2021). From the perspective of economic loss, in Africa alone, the cost of aflatoxin contamination is estimated to be more than $750 million, while the cost to African exporters is $670 million due to the restrictions on aflatoxin levels (Udomkun et al., 2017). In a review by Filazi and Sireli (2013), the occurrence of aflatoxins was 0 to 100% in cereals, 12.50 to 100% in oilseeds, 13.33 to 100% in spices , and 3.26 to 100% in dairy products, and affected every part of the world. Overall, about 4.5 billion people in the world (mostly in developing countries) are under the risk of exposure from aflatoxin contaminated crops (Filazi and Sireli, 2013).

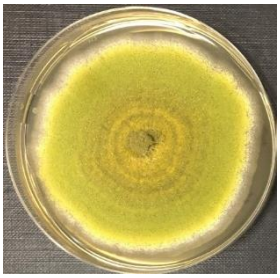
1.2 Introduction to *Aspergillus* sp.

1.2.1 Overview

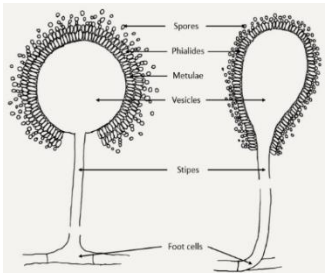
Aspergillus sp. is one of the most widely distributed fungi on crops in the world, some of which produce class 1 carcinogenic compounds – aflatoxins (Lee et al., 2014). Therefore, the mitigation of *Aspergillus* sp. growth and aflatoxin content has always been a major concern in food safety and security.

Aspergillus sp., a type of filamentous fungi, exist as spores or sclerotia in soil, and germinate as mycelia in substance, which then form conidiophores (Amaike and Keller, 2011). These fungi can be generally found in soil, air, decaying vegetation, and foods with high carbohydrate content (Mousavi et al., 2016). The distribution of *Aspergillus* sp. is spread by spores via air flow, soil movement, rain splash dispersal and insects or birds. Spores can protect fungi from extreme environment due to the thick wall, and help the fungi travel far and remain airborne for long time (Amaike and Keller, 2011; Lee et al., 2014).

Aspergillus flavus is an opportunistic pathogen of agricultural crops (Amaike and Keller, 2011). This species is characterized by fast-growing yellow green colonies (Fig. 1-1a), normally 75 to 80 mm in diameter after 7 days growth in the dark at 30°C on potato dextrose agar (PDA) (Liu et al., 2021). The length of the conidiophore stipe (Fig. 1-1b) is generally 400 to 800 µm, and the stipe is rough-walled. The vesicles are globose or elongated which is 20 to 45 µm in diameter. In addition, the spores (3 to 6 µm in diameter) are globose or ellipsoidal with roughened walls. There are also firm-walled structures with black globose or elongate shape called sclerotia formed from the same strain, which is generally 400 to 700 µm in diameter (Klich, 2007).



(a)



(b)

Fig. 1-1 Photo of *A. flavus* colony (a) and schematization of two morphological *Aspergillus* conidiophores adopted from Klich (2007) (b).

1.2.2 Factors affecting *A. flavus* growth

A. flavus can survive in a range of environmental conditions (Kosegarten et al., 2017). But its growth is affected by many factors, such as water activity (a_w) or relative humidity (RH) of matrixes, temperature, pH, protein concentration, fat concentration and gas composition in different food matrixes. Among them, a_w/RH and temperature are crucial factors (Lee et al., 2014; Casquete et al., 2017; Kosegarten et al., 2017). For instance, *A. flavus* was found to grow on ground *Nyjer* seeds when a_w was above 0.82 at the temperature between 20 to 35°C, yet the favorable a_w at 27°C was 0.9 to 0.98, and 0.9 to 0.94 at 35°C (Gizachew et al., 2019). In rice during post-harvest storage, *A. flavus* could grow at a_w 0.82 on brown rice at temperatures between 20 to 35°C, but could not grow on polished rice under the same conditions. The optimum conditions for fungal growth were a_w 0.92 at 25°C and 0.92 at 20°C on brown rice and polishes rice respectively (Mousa et al., 2013). For gas composition, the growth of *A. flavus* was inhibited with increasing CO_2 (Ayranci and Karaca, 2021). In contrast, pH, protein and fat concentration had less effect on *A. flavus* growth than other environmental factors, but the strength of effect of incubation conditions (a_w, temperature) on fungal growth strongly depends on pH, protein and fat concentration in culture medium (Kosegarten et al., 2017).

1.2.3 *Aspergillus* sp. infections

1.2.3.1 Pathogenicity in plants

Klich (2007) reviewed early studies on pathogenicity of *A. flavus* in plants: the ear rot of corn infected by *A. flavus* was reported in 1920; the cotton fibre infection called yellow spot disease was known in 1955, and the viability of infected cotton seeds was reduced by 60% after 30 years later; in peanuts, *A. flavus* not only caused necrotic lesions of seedlings or secondary roots, but also led to chlorosis on above-ground parts, lack of development of secondary roots and a rot of mature peanuts in the soil.

1.2.3.2 Aspergillosis

Aspergillosis is a human and animal condition caused by infection by *Aspergillus* sp. For the invasive infections, any organ can be infected target, but the respiratory tract was a key location for infections due to inhalation of spores (Arastehfar et al., 2021). The *Aspergillus* sp. can cause moderate allergies and severe asthma, while the infections were normally limited to the immunocompromised individuals or transplant patients (Chakrabarti et al., 2011; Mousavi et al., 2016). In terms of immune status of the host and the amount of spore exposure, the clinical course of pulmonary aspergillosis could be acute or chronic (Chakrabarti et al., 2011). *A. fumigatus* caused the majority of infection in pulmonary aspergillosis, whereas the bronchopulmonary infections caused by *A. flavus* is about 10% of total cases (Krishnan et al., 2009).

Central nervous system caused by *Aspergillus* sp. were rare but fatal conditions (Miceli, 2019). *A. flavus* may spread to the brain through infection of a primary lesion in nose or ear, or via hematogenous spread as a part of dissemination in an immunocompromised host (Rudramurthy et al., 2019). This invasion may lead to brain abscess, cerebritis, meningitis, cranial sinus thromboses, and ventriculitis, thus patients can appear several of symptoms including fevers, headaches, lethargy, altered mental status, seizures, abnormal gait, dizziness, or focal neurological findings (Miceli, 2019).

Apart from the aspergilloses mentioned above, *Aspergillus* invasion might also cause rare cardiac aspergillosis, cutaneous and subcutaneous aspergillosis and bone and joint infections (Chakrabarti et al., 2011; Rudramurthy et al., 2019). The common ground of these three aspergilloses was that most of the invasion occurs during or after surgery. Cardiac aspergillosis has been found to be associated with contaminated grafts, sutures, prosthetic valves or intra-operative dispersion of *A. flavus* spores (Pasqualotto and Denning, 2006; Chakrabarti et al., 2011).

1.3 Introduction to aflatoxins (AFs)

1.3.1 Overview

Aflatoxins (AFs) are secondary metabolites, which are mainly produced by *Aspergillus Flavi* (e.g. *A. flavus*, *A. parasiticus*, *A. nomius*), but not limited in these species (Kumar et al., 2016; Caceres et al., 2020). For *A. flavus*, species of all sclerotia size produce aflatoxin B_1 (Fig. 1-2a), B_2 (Fig. 1-2b), G_1 (Fig. 1-2c) and G_2 (Fig. 1-2d), while the species with sclerotia >400 mm in diameter can only produce aflatoxin G_1 and G_2 (Amaike and Keller, 2011). The B group is characterized by bifuran coumarins fused to cyclopentanone, and the G group by bifuran coumarins fused to a lactone (Gourama and Bullerman, 1995). On a thin-layer chromatographic plate, AFB_1 and AFB_2 show blue color of fluorescence under ultraviolet light, and AFG_1 and AFG_2 show green color; and the numbers (1 or 2) refer to the relative migration distance (Klich, 2007). Of the four aflatoxins, AFG_2 occurs in the largest quantity with the lowest toxicity. However, AFB_1 is the most toxic. It has been was classified as class I carcinogen by WHO (Bbosa et al., 2013). The potency of toxicity, carcinogenicity, mutagenicity for four aflatoxins is in the order of AFB_1> AFG_1> AFB_2> AFG_2 (Bbosa et al., 2013). Aflatoxins are not essential elements for fungal normal growth. It is believed that the advantages of producing aflatoxins might be a) as poisonous agents to protect from predators, b) unknown or not fully understood regulatory growth factors, or c) reserve products of various metabolic intermediates (Sinz and Shier, 2008).

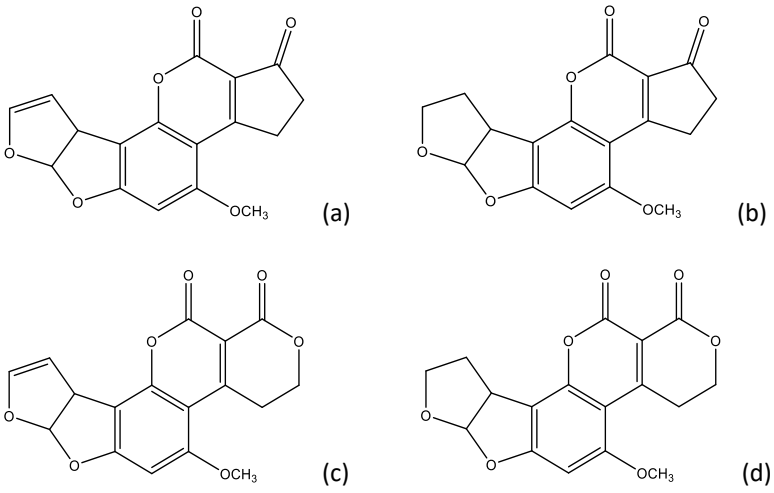


Fig. 1-2 Chemical structure of AFB_1 (a) AFB_2 (b) AFG_1 (c) and AFG_2 (d).

1.3.2 Biosynthesis of AFs

The process of AFB_1 synthesis is very complicated and not fully understood at the moment. Whereas current studies have demonstrated that the biosynthesis process involves more than 27 enzymatic reactions, and the genes coding of these enzymes are grouped in a cluster with their expression coordinated by 2 cluster-specific regulators: *aflR* and *aflS* (Caceres et al., 2020). This process has the quantity of oxidative rearrangements: a) monooxygenases joined incorporation of one oxygen atom to another reduced oxygen, b) dioxygenases joined ring-cleavage reactions, c) oxygen inserted between two carbons (Baeyer-Villiger reactions), d) attachment of functional groups (e.g. methyl, acetyl) joined by Cytochromes P-450 (Dutton, 1988; Caceres et al., 2020). The AFB_1 biosynthetic pathway is shown in Fig. 1-3. Same enzymes might be also involved in the biosynthesis of AFB_2, AFG_1 and AFG_2, while environment was the main factor affecting the production of different AFs by fungal strains (Yabe et al., 1988).

These results suggest that the same enzymes may be involved in the both biosynthetic pathways from ST to AFB1-AFG1 and DHST to AFB2-AFG2. The reactions described herein were not observed when the molds had been cultured in the YEP medium.

The AFs biosynthesis is affected by many factors. The optimum temperature of AFs production is normally 25 to 28°C depending to the substrates (liquid culture medium or cereals); meanwhile, the production of AFs is suppressed in liquid medium when the temperature <13°C or >42°C, and the AFs could not be detected <8°C or >37°C in same medium. Similarly, the incubation time of maximum AFs production was also related to the exhaustion of sugars in the medium and the onset of mycelial autolysis. In general, glucose, sucrose and fructose were the preferred carbon sources in AFs production, as well as essential single amino acids (glycine and glutamic acid) and essential minerals (zinc and manganese). Moreover, the minimum RH for AFs production ranged from 83 to 88%, and the production yield increased up to 99% with the raise of RH. In addition, AFs had a higher production yield at acid pH 4 to 6, and pH<6 favored AFB_1 and AFB_2, while pH>6 favored AFG_1 and AFG_2 (Gourama and Bullerman, 1995).

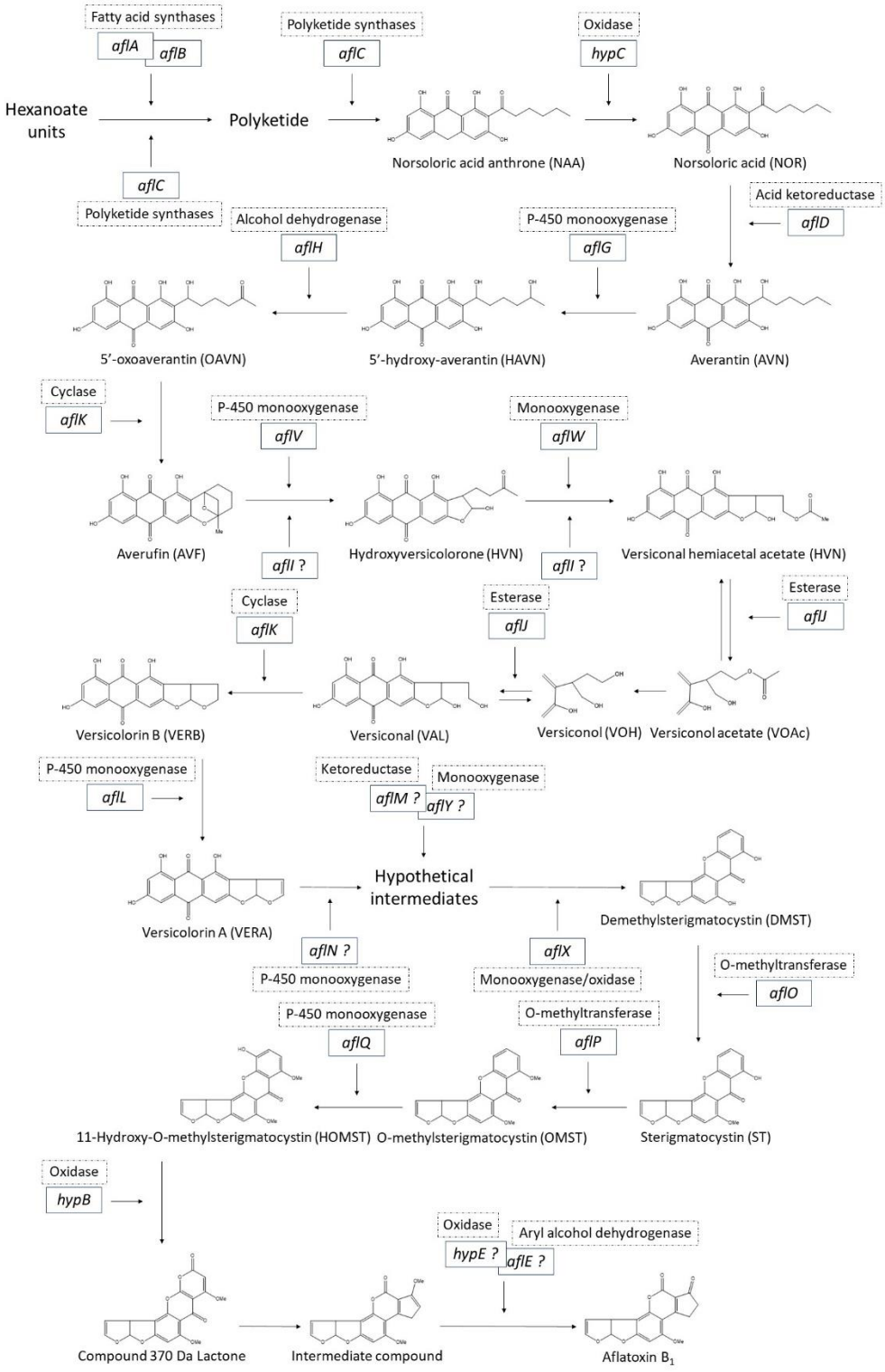


Fig. 1-3 Schematization of the main intermediates in AFB_1 biosynthetic pathway. '?' represents putative genes intervention belonging to the AFB_1 cluster adopted from Caceres et al. (2020).

1.3.3 Metabolism of AFs in human

There are two main pathways for AFs to enter human or animals: a) consuming and ingesting contaminated foods including AFM_1-contaminated dairy products, b) inhaling dust of AFs, AFB_1 in particular, from environment (Bbosa et al., 2013), with a) the primary route of exposure. After entering the body, these liposoluble compounds can be readily absorbed through the cell membranes of the respiratory and gastrointestinal tract into blood stream, and distributed to different tissues. Among them, liver, as the main organ of xenobiotics metabolism, is the major metabolic tissue (Larsson and Tjalve, 2000). There are various cytochrome P_{450} (CYP_{450}) enzyme isoforms in liver metabolizing AFB_1 to aflatoxin-8,9-epoxide (Fig. 1-4a, 1-4b), which may bind to DNA or protein and then causes DNA damage or liver cancer (Wild and Gong, 2010). CYP_3A_4 and CYP_1A_2 are the dominant CYP_{450} isoforms in human for AFB_1 metabolism, and both enzymes can catalyze the biotransformation of AFB_1 to the highly reactive exo-8,9-epoxide (Fig. 1-4a). Besides, CYP_1A_2 is also able to catalyze the epoxidation of AFB_1 that produce high yield of exo-8,9-epoxide, and hydroxylate AFB_1 to form a less toxic aflatoxin -- AFM_1 (Fig. 1-4c) (Guengerich et al., 1998). These are normally believed as the main metabolism pathway of AFB_1 in human body. Furthermore, in the hepatocytes, AFB_1 is also transferred to other metabolites by cytoplasmic reductase to such as aflatoxicol (Fig. 1-4d), and by microsomal mixed-function oxidases to compounds such as AFM_1, AFP_1 (Fig. 1-4e) and aflatoxin-epoxide, which might be deposited in different tissues and edible animal products (Bbosa et al., 2013). The metabolite AFQ_1 (Fig. 1-4f) converted by CYP_3A_4 has very little toxicity and can be excreted from body with urine (Wang et al., 1998).

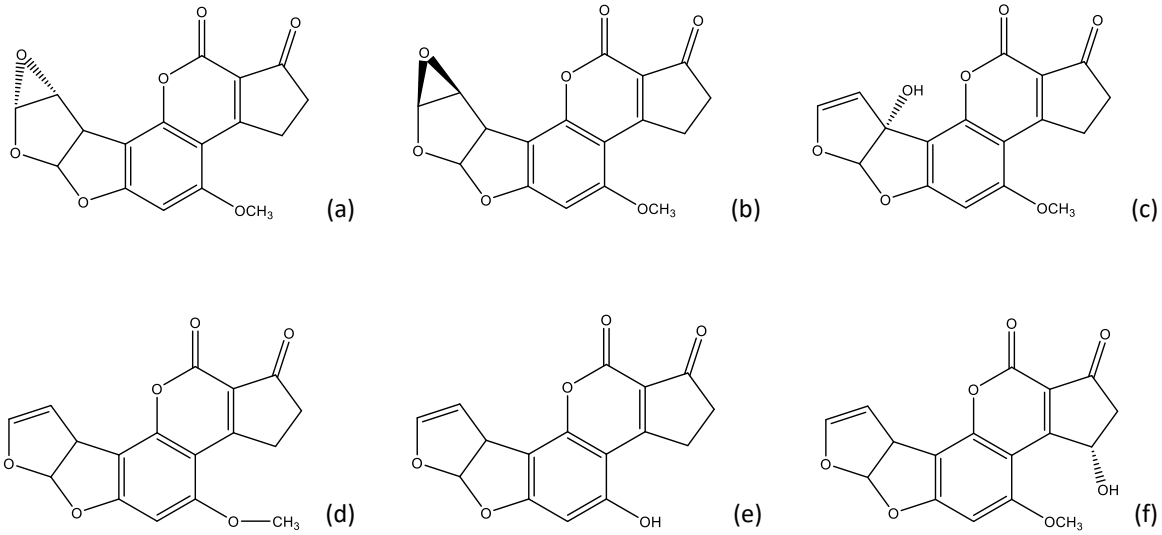


Fig. 1-4 Chemical structure of AFB_1 metabolized products: exo-8,9-epoxide (a), endo-8,9-epoxide (b), AFM_1 (c), aflatoxicol (d), AFP_1 (e) and AFQ_1 (g) (Guengerich et al., 1998).

1.3.4 Aflatoxin health risk

The aflatoxicosis usually occurs after an acute expose to moderate to high levels of AFs, AFB_1 in particular. The typical acute symptoms might involve hemorrhage, acute liver damage, edema, change in digestion, absorption or metabolism of nutrients, and even death (Bbosa et al., 2013).

Low-dose but long-time exposure to AFs is the most significant reason for the induction of hepatocellular carcinoma (liver cancer), and AFB_1 is the most hepatocarcinogenic compound in human and animals (Bbosa et al., 2013; Peles et al., 2019). In addition to liver cancer, malignancies of esophagus, trachea, kidney and lungs, carcinoma of the pancreas and osteogenic sarcomas have been indicated to be related with AFs exposure (Yilmaz et al., 2018). Besides, the formation of exo-8, 9-epoxide by AFB_1 epoxidation is an important process in genotoxicity (Bbosa et al., 2013). Either AFB_1 or the epoxide binding to DNA can cause a functional changes of DNA conformation (Raney et al., 1993). The exo-8, 9-epoxide is easily able to bind with guanine bases in DNA and form afltoxin-N7-guanine that results in transversion mutations in DNA (from guanine to thymine). This mutation affects the expression of hepatocellular carcinoma suppressor gene *TP53*, which is an important gene in prevention of DNA mutations or signalling apoptosis in cell life cycle (Wild and Turner, 2002; Bbosa et al., 2013).

Hepatocellular carcinoma (HCC) is the third leading reason of cancer deaths globally. It is

estimated that around 550 to 600 thousand new HCC cases prevail worldwide every year, and about 25 to 155 thousand of them were caused by AFs (Liu and Wu, 2010). The known carcinogenic mechanism is the accumulation of DNA adducts formed after AFs interacted with DNA, bringing a promising risk to the tissue (Bbosa et al., 2013). Moreover, the incidence of liver cancer raised with the increase in the infection of chronic hepatitis B (HBV) due to the damage to the liver by synthesis interaction of AFs and HBV. These interactions might include a) the aflatoxin-induced mutation fixated at the site of liver regeneration and hyperplasia formed by chronic HBV infection, b) DNA damage by AFs easily occurred on HBV-infected hepatocytes, c) an increasing susceptibility of chronic HBV infection in AF-exposed individuals, and d) an exacerbation of oxidative stress at co-exposure with AFs and HBV infection (Kew, 2013).

Although the Global Burden of Disease Injuries and Risk Factors Study has not listed aflatoxin exposure as a reason of childhood stunting due to lack of data, a growing number of studies have shown the link between the exposure and growth impairment in child early life (Gong et al., 2004; Mupunga et al., 2017; McMillan et al., 2018; Rasheed et al., 2021). It is reported that both weight and height of the first-year child decreased 0.8 kg and 2 cm respectively, when the aflatoxin albumin (AF-alb) in maternal blood increased about 10 times (from 10 to 110 pg/mg), which indicated that aflatoxin had a direct toxic effect *in utero* presenting the growth faltering of early children (Turner et al., 2007). The children stunting induced by aflatoxin might be associated with changes of immune function or malnutrition resulting from malabsorption, micronutrient deficiencies, impaired immune function, and vulnerability to gut infections due to intestinal integrity and hepatic metabolism (Rasheed et al., 2021). Because the contamination of foods is widespread in Sub-Saharan Africa, children in these countries are placed in a high risk of exposure to AFs (Mupunga et al., 2017).

Despite huge progress in the field of *Aspergillus* sp. and aflatoxin decontamination, the loss and health issues associated with contamination continue to cause problems across the world. Research into new approaches that complement traditional and established methods of aflatoxin control are needed. This thesis proposes two new cheap, sustainable and effective approaches for inhibition of *A. flavus* and mycotoxin reduction with a focus on AFB_1.

1.4 Aim, objectives and method outlines of project

1.4.1 Aim

The aim of this thesis was to investigate new cost-effective and environmentally friendly biological approaches to reduce aflatoxin contamination through *A. flavus* inhibition or AFB_1 removal based on utilisation of agricultural by-products as antifungal agents or toxin adsorbents.

1.4.2 Objectives

Chapter 2:

- Critically evaluate published literature on post-harvest approaches for fungal and aflatoxin decontamination of foods.
- Discuss the feasibility of different approaches to be upscaled from laboratory to industrial scales in different food matrixes.

Chapter 3:

- Investigate the antifungal activity of citrus peel extracts prepared using green extraction technologies.
- Isolate and characterise the antifungal compounds present in citrus peel extracts.

Chapter 4:

- Investigate AFB_1 adsorption ability by corn by-products in a liquid model system.
- Understand the adsorption interaction from the perspective of theoretical models, and preliminarily explore the adsorption mechanisms.

Chapter 5:

- Discussion of the proposed mechanisms behind the antifungal and binding effects, potential implications for food safety and utilisation of these approaches in practice.

1.4.3 Outlines of methodology

Chapter 2:

- A critical literature review was conducted for English language peer-review articles indexed in Google Scholar, PubMed, ScienceDirect and EMBASE databases (updated March 2020) with the addition of other scientific reports in web sources or in previously published reviews. to the search terms were 'Fungi inhibition', 'Antifungal activity', 'Fungal decontamination', 'Mycotoxin occurrence', 'Mycotoxin reduction', 'Mycotoxin detoxification' and 'Mycotoxin degradation'. All the decontamination approaches were classified depending on if additional chemicals were added into the system (yes, physical approaches; no, chemical approaches), and if the additional chemical obtained from biological materials (yes, biological approaches; no chemical approaches).

Chapter 3:

- Antifungal assay: the agar dilution method was carried out for the study of fungal growth inhibition in this study. The method involved the direct addition of certain/different concentrations of the extracts into Potato Dextrose Agar (PDA) followed by the inoculation of a fungal disc to the surface of the agar plate. Growth was assessed after incubation for a defined period of time (7 d). The agar dilution method is accurate, easily reproduced and low-cost. Therefore, this method is one of the most commonly used techniques to determine the inhibition activity of antifungal agents.
- Solid phase extraction (SPE): the SPE was applied to the isolation of polyphenol from the extracts. SPE is a rapid and effective way to prepare and purify one or more analytes from a liquid sample through extraction, partition and adsorption by a solid stationary phase. The result was that the undesired ingredients were washed off (washing fractions), and the desired analytes were retained on the stationary phase, which was finally eluted and collected with an appropriate eluent.
- Determination of total phenolic content (TPC): the Folin-Ciocalteu method was performed for the TPC determination of extracts. The Folin-Ciocalteu reaction is an electron transfer based assay, which gives reducing capacity that expressed as phenolic content. It has been widely used in the determination of TPC in plant-derived food samples.

- Determination of total flavonoid content (TFC): the $AlCl_3$ assay performed for the TFC determination of extracts. This spectrophotometric assay bases on aluminium complex formation with different classes of flavonoid family. Similarly, it has been widely used in the determination of TFC in plant-derived food samples.
- Polyphenol quantification: polyphenols in extracts were quantified by high-performance liquid chromatography (HPLC) system. HPLC is a technique in analytical chemistry used to separate, identify, and quantify each component in a solution. Based on the relative affinities of different molecules in the mobile phase and the stationary phase, polyphenols were retarded while passing through the column (stationary phase), so that distinct polyphenols could get eluted at different retention times. Whereafter, a UV detector was giving signal intensity by measuring content of each component.

Chapter 4:

- Adsorption experiment: a batch adsorption experiment from liquid phase was performed to measure adsorption ability and adsorption equilibrium. It consisted of the addition of a known amount of adsorption to a fixed volume of AFB_1 solution at different initial concentrations under various conditions.
- Model studies: kinetic study of adsorption was carried out to describe the reaction rate of the process and adsorption capability of the adsorbents. Isotherm models were applied to describe the maximum adsorption capability of AFB_1 adsorbed on CCP and molecules under different initial concentration at constant temperature. In addition, the thermodynamics study provided theoretical evidence of feasibility and spontaneity of adsorption reaction.
- AFB_1 quantification: the unbound AFB_1 was quantified by HPLC with a fluorescence detector. This detector measured light emission from excited atoms in AFB_1 to obtain the fluorescence intensity.

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Chapter 2: A Review of Post-harvest Approaches to Reduce Fungal and Mycotoxin Contamination of Foods

Abstract: Contamination of agricultural and food products by some fungi species that produce mycotoxins can result in unsafe food and feed. Mycotoxins have been demonstrated to have disease-causing activities, including carcinogenicity, immune-toxicity, teratogenicity, neurotoxicity, nephrotoxicity and hepatotoxicity. Most of mycotoxins are heat stable and cannot be easily destroyed by conventional thermal food processing or domestic cooking methods. Post-harvest approaches to prevent mycotoxin contamination and detoxify mycotoxins from contaminated food are important topics in food safety research. Physical, chemical, and biological methods have been applied to prevent fungal growth or mycotoxin production, or to reduce mycotoxin content in the postharvest period and contribute toward mitigating against the effects of mycotoxins on human health. This literature review aims to evaluate postharvest approaches that have been applied to control both fungi growth and mycotoxin content in food and discuss their potential for upscaling to industrial scale.

Key words: Antifungal, contamination, food safety, fungi, mycotoxin, postharvest, prevention or mitigation approaches, reduction

2.1 Introduction

Agricultural and food products can be contaminated by fungi, most particularly during the post-harvest period. Some fungi can produce toxic metabolites, named mycotoxins, which have a negative impact on the safety of food and feed. Dietary exposure to mycotoxins causes health issues due to their biological activities, which include carcinogenicity, immune-toxicity, teratogenicity, neurotoxicity, nephrotoxicity and hepatotoxicity (Dalié et al., 2010; Jard et al.,

2011). Some of these toxicities can be acute (WHO, 2015; WHO, 2017), resulting in illness or death within a few days of exposure to heavily contaminated food. Meanwhile, mycotoxins can have cumulative effects at lower doses, resulting in chronic health effects that manifest over several months or year (Tola et al., 2016).

More than 100 fungi species have been found to produce over 400 poisonous metabolites (Jard et al., 2011). The most common agricultural mycotoxins comprise aflatoxins (AFB_1, AFB_2, AFG_1, AFG_2 and AFM_1), fumonisins (FB_1, FB_2), ochratoxin A (OTA), the trichothecene mycotoxins (type A: T-2 and HT-2, type B: deoxynivalenol (DON), nivalenol (NIV)), and zearalenone (ZEN), patulin (PAT) and egot alkaloid. Minor mycotoxins include cyclopiazonic acid, sterigmatocystin, gliotoxin, citrinin and citreoviridin. Mycotoxins are produced primarily by *Aspergillus* sp., *Penicillium* sp., *Fusarium* sp. and *Claviceps* sp. (CAST, 2003; Hathout and Aly, 2014; Petruzzi et al., 2014; Schaarschmidt and Fauhl-Hassek, 2018).

Mycotoxin producing-fungi are prevalent worldwide. According to previous report published in 2011, more than a quarter of the world's agricultural products are heavily contaminated by mycotoxins at levels above the European Union (Table. 2-1) and *Codex Alimentarius* limits (Jard et al., 2011) . However, a more recent report indicates that mycotoxins are detected in 60 to 80% of agricultural products. The increase is likely due to a combination of the improved sensitivity of analytical methods and impact of climate change (Eskola et al., 2019). Moreover, more than 50% of food products are showing co-occurrence of more than one mycotoxin (BIOMIN, 2015). Mycotoxin contamination may occur during pre- and/or postharvest periods. The occurrence of mycotoxins in different crop products is shown in Table. 2-1. It appears that cereals (such as wheat, maize, rice, barley, and sorghum) are the most commonly contaminated products, although mycotoxins can also be found in animal products (meat, eggs, and milk), pulses, oilseeds, dry fruits, and nuts. The most important agricultural pathogens are *Aspergillus*, *Fusarium*, and *Penicillium* sp. *Aspergillus* sp. exists in warm (25 to 42°C) environments, which can be humid or dry (even down to -35 MPa water potential). These conditions are common in soil, storage areas and manufacturing facilities (Klich, 2007; Tola et al., 2016). In *Aspergillus* sp., the production of aflatoxins is related to spore production (Klich, 2007; Tola et al., 2016). In temperate regions,

Aspergillus sp. also contributes to OTA production. *Penicillium* sp. can produce ochratoxins at temperatures as low as 5℃ (Tola et al., 2016).

As some mycotoxins are highly toxic, maximum limits (MLs) standards have been established to protect the consumers' health by avoiding excessive harmful mycotoxins in foods. In the early 21st century, approximately 100 countries in the world (covering about 85% of inhabitants) have set MLs to regulate the maximum amount of mycotoxins permitted in human and animal feed (van Egmond et al., 2007). The MLs of main mycotoxins set by the European Commission are shown in Table. 2-1. However, these limits exert an impact on the agricultural export market, where least contaminated crops are exported to generate income, whereas more contaminated foods may be traded in the producing country, especially in low-income countries where regulations are poorly enforced. As a result, it is critical for the food and economic security of low-income countries to reduce fungal and mycotoxin contamination of foods.

Because of the different distinct hazards caused by fungi (microbiological) and toxins (chemical), the risk control strategies should be addressed simultaneously and where possible synergistically. Prevention of fungal growth is usually considered as an early step during production and postharvest storage. If fungal growth cannot be avoided, approaches to decontaminate the food of the toxin through processing must be considered.

This is a comprehensive review of postharvest approaches that have been applied to reduce fungal growth and mycotoxin contamination in foods. The review includes a comparative evaluation of the efficacy of different approaches, including physical, chemical, biological, and their combination, on fungal growth and mycotoxin content. The review discusses the feasibility of these different approaches to be upscaled from laboratory to industrial scales within different food systems.

Table. 2-1 The occurrence of main mycotoxin contamination in raw and processed products and regulation of mycotoxins in European Communities

Mycotoxins	Products		European Communities standards (ng/g)	Reference
Aflatoxins	Raw products	Almonds, pistachios and apricot kernels,	10 (for human direct consumption)	de Medeiros et al. (2012) Milani and Maleki (2014) Calado et al. (2014) Eskola et al. (2019) Cinar and Onbaşı (2019) EU (2006)
		Oilseeds	15 (for oil production)	
		Cereals	10 (for processing)	
		Spices	10 (for human direct consumption)	
		Milk	Cannot be detected	
	Processed products	Dried fruits, copra	4 (for human direct consumption)	
		Maize grits	Cannot be detected	
		Cheese	Cannot be detected	
Fumonisins	Raw products	Germ, bran, rice, sorghum, legumes, cowpea seeds, triticale,	4000	de Medeiros et al. (2012) Milani and Maleki (2014) Calado et al. (2014) Eskola et al. (2019) Cinar and Onbaşı (2019) EU (2006)
		Maize	1000 (for human direct consumption)	
		Asparagus	NM	
		Milk	NM	
	Processed products	Grits, maize-based products, wheat flour	800 (for adult direct consumption)	
		Beer	200 (for human direct consumption)	
Deoxynivalenol	Raw products	Cereals	750 (for human direct consumption)	de Medeiros et al. (2012) Milani and Maleki (2014) Eskola et al. (2019) Cinar and Onbaşı (2019) EU (2006)
	Processed products	Wheat flour	750 (for human direct consumption)	
		Bread, pasta, pretzel, cookie	500	
Ochratoxin A	Raw products	Cereals, legumes, coffee beans, nuts, pulses, sesame seeds,	5 (for processing)	Larsen et al. (2001) Varga and Kozakiewicz (2006) Eskola et al. (2019) Cinar and Onbaşı (2019) EU (2006)
		Spices	15 (for human direct consumption)	
		Apples, peaches, strawberries, pears, oranges, figs, mangoes, tomatoes, watermelons, yam, potatoes, garlic, onions,	NM	
		Milk, eggs, meat	NM	
	Processed products	Grape juices, wine vinegar	2.0 (for human direct consumption)	
		Breakfast cereals & snacks	3.0 (for human direct consumption)	
		Infant cereals	0.5 (for infant direct consumption)	
		Bread, pasta	0.5 (for human direct consumption)	
		Flour	0.5 (for human direct consumption)	
		Cocoa	5	

		Dried vine fruits	10	
		Sausage	NM	
		Cheese, milk-based products	0.5 (for human direct consumption)	
		Bottled water, plant food supplement, food colouring agent	NM	
Zearalenone	Raw products	Maize	350 (for processing)	de Medeiros et al. (2012) Calado et al. (2014) Eskola et al. (2019) Cinar and Onbaşı (2019) EU (2006)
		Cereals, sesame, soy beans, nuts	75 (for human direct consumption)	
	Processed products	Cereal-based products	50 (for adult direct consumption)	
Patulin	Raw products	Wheat straw residue	NM	CAST (2003) EU (2006)
	Processed products	Fruit juice	25 (for adult direct consumption)	

NM: Not mentioned

2.2 Control of fungal growth and mycotoxin production

The most effective way to reduce the mycotoxins in the food chain is to prevent the fungus growing in the first place, and if fungi do happen to be present in the food, then to prevent the toxin from being produced. A range of physical, chemical, and biological approaches have been applied, both at industrial and laboratory scales.

2.2.1 Physical approaches

2.2.1.1 Temperature and humidity control

Storage of crops causes a mini ecosystem containing the biotic factors (crops, microorganisms, etc.) and abiotic factors (water, air, temperature, etc.) suitable to fungal growth (Peng et al., 2018). Similar to other living organisms, fungi require water and an optimal temperature to survive and thrive. Moisture content (MC) and storage temperature can be controlled to be outside of the microorganism's optimum to reduce metabolic activity and decreased growth. Moreover, water content and temperature are one of the easiest factors to control during food storage, at both industrial and domestic scales. Although both relative humidity (RH) and MC are used to reflect the water content of food, it is better to use equilibrium RH, because the impact of equilibrium RH on spoilage organisms is consistent across different foods, regardless of their composition (Bradford et al., 2018). When the equilibrium RH is below 65%, microorganisms stop growing, meaning food is safe for at least 1 year of storage at ambient temperature. Storage temperature can also contribute to crop longevity (Bradford et al., 2018). In stored rice, both raising temperature (10 to 40℃) and RH (12 to 98%) significantly increased the growth of both *Aspergillus* sp. and *Penicillium* sp. by about 4 to 6 log colony forming units per gram (CFU/g) from about 3.8 log CFU/g. According to the result of multiple linear regression analysis, changing one unit of temperature resulted in stronger impacts on fungal populations than changing humidity (Mannaa and Kim, 2018). Additionally, Choi et al. (2015) showed that both 21℃ with 97% humidity and 30℃ with 85% humidity were associated with an increase of population of *A. flavus* by about 3 log CFU/g and the production of aflatoxins during the 120-day storage period, whereas when the rice was stored at 21℃ with 85% humidity, *A. flavus* population could be constant and

no aflatoxins were produced. For *F. graminearum*, 97% humidity encouraged the fungal growth from 2.5 to 4 log CFU/g at 21℃. When the humidity was reduced to 85%, *F. graminearum* and DON production could be controlled. Thus, dry (below 85% RH) and low temperature (below 21℃) are good strategies for controlling fungal growth. However, these conditions may be difficult to maintain in warm and humid countries where a refrigerated and ventilated store may not be available. Moreover, vegetable foods tend to respire, causing moisture and temperature rises during postharvest storage, and thus the environmental conditions must be regularly monitored.

2.2.1.2 Modified atmosphere treatment

Modified atmosphere (MA) approaches include modification of the gas composition (e.g. O_2, CO_2, N_2) considering temperature, RH, MC and competing microorganisms (Magan and Olsen, 2004), and is usually applied for fresh food preservation (Bouletis et al., 2016; Putnik et al., 2017; de Siqueira Mendes et al., 2019). Fungi have a different sensitivity to atmosphere compositions. In general, high CO_2 and low O_2 content can contribute to inhibition of foodborne fungi. *P. roqueforti* and *A. flavus* could not grow in both 40% and 60% CO_2 environments balanced with N_2 and less than 0.5% O_2, but weakly grow (about 30 mm in 30-day incubation) in 20% group CO_2 (Taniwaki et al., 2009). MA at a large scale can be expensive, and there may be issues in displacing and replacing the gases during the storage period.

Modified atmosphere package (MAP) is a strategy that controls gas composition immediately surrounding the food within gas-impermeable packaging. Wheat and rye bread artificially inoculated with several fungi were packaged with 0%, 50%, 75% or 100% CO_2, 1%, 0.03% O_2 or in the presence of O_2 absorber, and balanced with N_2. Notably, the gas composition would be changing during the storage of the bread. MAP was more effective against fungal growth on rye bread, as fewer fungi grew with the increase of CO_2. But for *P. roqueforti*, this main contaminant of rye bread was inhibited only in the presence of O_2 absorber. For wheat bread, the most resistant to CO_2 was *P. commune* which could grow in 99% CO_2. *A. flavus* grew in the lowest O_2 and 75% CO_2 (Suhr and Nielsen, 2006). So far, MAP has become a widely used way of food preservation because of its efficiency, convenience and safety. It is cheaper and easier than large-scale MA as it is only necessary to fill the packaging with modified gas. MAP requires suitable

packaging, which is generally plastic based, and will inevitably cause usage and disposal of a large number of plastic packaging.

2.2.1.3 Irradiation treatment

Irradiation of food for safety is based on the utilization of ionizing energy to inactivate microorganisms by changing their cellular structure or physiological functions, including DNA strand breakage, cell membrane rupture/leakage, or mechanical damage of cell walls (Calado et al., 2014). The effectiveness of the irradiation method depends on many factors, such as irradiation dose, the genetic microbial attributes (e.g. morphological structures, physiological stage) and the environmental condition of the irradiated materials (e.g. temperature, pH) (Magan and Olsen, 2004).

Aziz et al. (2007) treated wheat, maize, and barley collected from Cairo (Egypt) markets with gamma-irradiation and evaluated the occurrence of four *Fusarium* strains and FB_1 production. At 5 kGy, both *Fusarium* sp. counts and FB_1 production on barley samples were completely decontaminated. Same results could be observed on wheat and maize under 7 kGy irradiation. When the dose was below 5 kGy for barley or 7 kGy for wheat and maize, the growth of *Fusarium* sp. and production of FB_1 could be inhibited up to 85% and 97% respectively. Similar observations were later reported by Akueche et al. (2012) who studied the effect of gamma-radiation treated on sesame grains sampled from Abuja (Nigeria) markets. In this study, 135 fungal strains including *Aspergillus* sp., *Penicillium* sp., and *Fusarium* sp. were isolated from nonirradiated sesame grains. But only 34 strains were found on grains after 3 kGy gamma-irradiation, and no of fungal species was found on grains irradiated between 6 to 15 kGy.

In an experiment aimed at preventing fungal infection in fruits and vegetables, fungi were usually artificially inoculated on the fruits or vegetables, and then treated with various dose of irradiation. In general, the results showed better fungal inhibition comparing to control group with the increasing irradiation dose in peppers, oranges, broccoli, cabbage, tomato, bean sprout and papaya (Bari et al., 2005; Cia et al., 2007; Yoon et al., 2014; Jeong et al., 2016). The irradiation could not only reduce the fungi, but also affected the production of mycotoxins. For instance, the total fungi isolated from packed hot peppers were 4.8×10^3 CFU/g, total *Aspergillus* count were

4.7×10^2 CFU/g and aflatoxin level was 1.14 ppb on average. After 2, 4, 6 kGy irradiation treatment, over 90% fungi could be reduced. But only a nonsignificant reduction of 6% on aflatoxin levels was observed at 6 kGy gamma radiation (Iqbal et al., 2012). In addition, as the fruits and vegetables tend to easily lose their sales value, the effect of irradiation treatment on product qualities should be considered. In a study of Bari et al. (2005), appearance, texture, colour, taste and overall acceptability were used as sensory indicators to determine the quality of broccoli, mung bean sprouts, cabbage and tomato in both untreated and treated groups. Of all indicators, texture was the worst affected parameter after irradiation treatment in the four tested vegetables, and the sensory evaluation of other indicators gradually got worse with increasing dose (maximum 1.0 kGy). Despite this, with 1.0 kGy, less than 7-days storage was acceptable for each vegetable at refrigeration temperature.

The primary advantages of irradiation are non-residual and high efficacy, so that it can be considered as environment friendly mycotoxin reduction approach. Nevertheless, the nutrient loss, high costs and secondary products of uncertain safety in treatment are not negligible as well (Calado et al., 2014), added to the deterioration of sensory quality that can be cause by irradiation.

2.2.2 Chemical approaches

2.2.2.1 Control by chemical antifungal agents

In general, many antifungal agents are low-molecular-weight organic acids and their salts (Magan and Olsen, 2004), and some of them are applied as food additives (SANTE, 2017). Marín et al. (2000) revealed that *Penicillium* sp. had the highest sensitivity to both 0.5 and 1.0 g/kg propionates than *Aspergillus* sp. and *Fusarium* sp. at 25℃ in culture medium. The efficacy of propionates was higher at 7 days rather than at 14 to 21 days. In another study, Valencia-Chamorro et al. (2008) screened 15 chemicals and their mixture in hydroxypropyl methylcellulose-lipid edible composite films on the effects of fungal growth. Among the chemicals, sodium bicarbonate, potassium sorbate (2%), sodium benzoate (2.5%), sodium salt of

methyl paraben (1%/1.5%), sodium salt of ethyl paraben (1%) and sodium salt of propyl paraben (1%) and the mixtures of potassium sorbate (1.5%) with sodium propionate (0.5%), sodium benzoate (2%) with potassium sorbate (0.5%) and sodium benzoate (2%) with sodium propionate (0.5%) displayed the inhibition on growth of *P. digitatum* and *P. italicum* at all concentrations (10^3, 10^4 and 10^5 spores/mL). The inhibitory effects were dose dependent. Sodium salt of methyl paraben at the concentration of 1.5% showed the best performance, whereas no synergistic effect could be found in the mixtures of two antifungal agents. This edible coating displayed potential application prospects. In a recent research, a novel material, zinc oxide slightly coated with sliver nanoparticles, was demonstrated to inhibit the growth of *A. niger* (Tornero et al., 2018). Coating is one of the popular methods to preserve fresh fruits and vegetables. The coatings inhibit respiration, delay softening, and colour changes via controlling the internal gas composition and water vapor (Conforti and Totty, 2007; Mehyar et al., 2014). When fungal inhibitors are added into the coatings, the counting of not only mycotoxin-producing fungi but also other spoilage microorganisms can be significantly reduced. For example, Salas-Méndez et al. (2019) compared antifungal effect of control group, edible nanolaminate coating (synthesized by the aminolysis of polyethylene terephthalate) (NL) and nanolaminate coating with added an extract from *Flourensia cernua*, a Mexican endemic plant growing in arid and semiarid areas (NL + FcE). Fungi could be found from the beginning of storage in control and NL group at 20℃, whereas NL + FcE coating could prevent the fungal infection for 6 days. On the 15th day, the counting of fungi and yeasts in control group was about 1,000 times than those in NL + FcE group, and was about 100 times than those in NL group. Nevertheless, in a report of Mehyar et al. (2014), the coating of date palm cultivar with pea starch + carnauba wax and zein protein + carnauba wax could only reduce fungi and yeast about 1 log CFU/g after 14 days, but the coatings lost their effect in third week at 25℃. Antifungal agent treatment, with or without coating, is of low cost and easy to use control approach, but the safety of the remaining fungicide residues in the treated products is also a major concern and this has highlighted the necessity of using antimicrobial compounds that are safe to humans and animals. Antifungal agents also tend to lose their effectiveness over time, putting into question their application for large-scale crop and food storage.

2.2.2.2 Photodynamic treatment

Photodynamic treatment is a method that utilizes the interaction of the nontoxic photosensitizer and a particular wavelength of visible light (Al-Asmari et al., 2018). This therapy is mainly used in oncology, ophthalmology and dermatology (Preuß et al., 2014). In recent years, the photodynamic treatment has been investigated for antimicrobial properties, as the photosensitizer, induced by light of specific wavelength, generate cytotoxic substances that cause biochemical and functional disturbances of the cell membrane component and leads the damage to the microbial cells (Temba et al., 2016a; Al-Asmari et al., 2018). Curcumin is one of the most common photosensitizers in photodynamic studies. In Temba et al. (2016a) study, about three magnitudes of log of *A. flavus* spores counts were reduced by 84 J/cm^2 irradiation at 420 nm with both 15 and 20 µM of curcumin. When 5 log CFU/mL of spores were spiked into whole maize kernels, 1.9 log CFU/mL of spores were decreased at 60 J/cm^2 light with both 25 and 45 µM of curcumin, while 2.8 log CFU/mL of spores were reduced in milled kernels under same conditions. In another Temba et al. (2019) study, the effect of pH and temperature on *A. flavus* elimination under the reaction condition of 100 µM curcumin stock solution with the irradiation 420 nm wavelength at 60 J/cm^2 was investigated. Compared to nonilluminated group, the *A. flavus* spores in illuminated group were about two magnitudes lower at pH 1.5 to 9, and showed a sharp decrease at pH 12.5 in both groups. Similar pattern could be found in hyphae reduction. In the temperature-depending assay, although the counts in nonilluminated group were still higher than those in illuminated group, temperature (from 15 to 45℃) did not have significant influence on *A. flavus* spores and hyphae. In addition, about 66.7% of produced AFB_1 was not detected under the light treatment with curcumin stock solution. Njoki et al. (2017) reported 6 plants extracts (*Solanum aculeastrum, Syzygium cordatum, Prunus africana, Ocimum lamiifolium, Lippia kituiensis*, and *Spinacia oleracea*) could inhibit the growth of colony of *A. flavus* 4 to 47 mm (up to 42%) at concentration of 450 and 600 mg/mL. However, when *A. flavus* were treated with the increasing treatment dose and time of visible light (420 nm), the fungi were inhibited up to 95% at same concentration of plants extracts. Preuß et al. (2014) synthesized new photosensitizers and observed the prevention of growth of *A. niger* and *P. purpurgenum*. Besides, the new synthesized photosensitizer inactivated germination of conidia. As a novel method, the

photodynamic treatment showed a potential prospect to control mycotoxin-producing fungi. However, current studies mainly focus on fundamental research at laboratory scale, whereas the future research could consider the safety of photosensitizers and photolysis products and the application of photodynamic treatment in real and large-scale food systems.

2.2.2.3 Electrolyzed oxidizing water treatment

Electrolyzed oxidizing water (EOW) is obtained from electrolyzed NaCl solution, transforming water molecules and chloride ions into chlorine oxidants (Cl_2, HOCl/ClO−) that show antimicrobic properties. EWO contains two types of water: strongly acidic EOW and neutral electrolyzed water (NEW). The antimicrobial effect mainly depends on the level of ·OH. The radicals can break the normal morphological structure of spores, and is closely related to the damage of conidium cell wall and membrane, which leads to the spores lose their normal function. NEW is non-toxic and safe to human, it can be applied to fungi decontamination (Guentzel et al., 2010; Xiong et al., 2010; Gómez-Espinosa et al., 2017). Okull and Laborde (2004) used EOW to inactivate 1 to 4 magnitudes of *P. expansum* spores depending on the concentration and exposure time. In the apple infection test, the apples were inoculated with 10^6 CFU/mL of spores and treated with 50% and 100% EOW for 5min, and stored at 25℃ for 6d. In nontreated apples, once wounds were infected by spores, the decay was inevitable (100% incidence). But when treatments were applied, decay in apple was only 18.4% for 50% EOW and 10.2% for 100% EOW. Therefore, the use of EOW can be considered as a potential method in an apple cleaning system. Xiong et al. (2010) compared the elimination of *A. flavus* by both EOW and acidic EOW. The results illustrated the population of spore survival treated by acidic EOW was 5.77 log conidia/mL, which was 1.48 log conidia/mL less than control group, and no spores could be found in EOW group. This is because that EOW showed a stronger signal on ·OH level than acidic EOW. With the addition of mannitol (a radical scavengers) in reaction system, the survival population was increased, which also provided a strong evidence that ·OH played the most important role in the inhibition of *A. flavus* spores activity.

2.2.2.4 Plasma treatment

Plasma is an ionized gas, with zero net electrical charge, that can be induced in any neutral gas at particular pressure and temperatures conditions. Examples of natural plasma are sun and polar gases, whereas artificial plasma include dielectric barrier discharges plasma, microwave plasma, inductively coupled plasma, radio frequency, and commercial ozone (Misra et al., 2019). These plasmas could inactivate a variety of mycotoxin producing fungi on a range of foods, including fruits, vegetables, herbs, spices, cereals, nuts, and meat products in seconds. However, the effect of plasma on food quality depends on the type of plasma, the duration of treatment, and plasma intensity (Misra et al., 2019).

Among the cold plasmas, ozone is one of the best-documented plasma on antifungal activity. This strong oxidant can progressively oxidise unsaturated lipids in the microbial membrane or cellular proteins, leading to a leakage or rapid death of the cell (Freitas-Silva and Venancio, 2010). In addition, ozone can reduce conidia germination (Savi et al., 2015a). *A. flavus* artificially spiked on wheat was reduced by up to 96.6% with the 60 µmoL/moL O_3 gas treatment for 120 min and 100% with the same concentration for 180 min (Savi et al., 2015a), *P. citrinum* as well, whereas *F. graminearum* was more sensitive to the same concentration of O_3 gas, inhibited by up to 96.81% in 30 min and completely inhibited in 180 min (Savi et al., 2015a). Naturally occurring *Aspergillus* sp. and *Penicillium* sp. on rice could only reduce up to 70% in short-time treatment (30 min) at 10 mg/L O_3 gas (Beber-Rodrigues et al., 2015). Moreover, O_3 treatment can also decrease mycotoxin production. In one of above studies, produced AFB_1 degraded 69.5% and 72.2% exposed under 40 and 60 µmoL/moL of O_3 gas respectively for 180 min (Savi et al., 2015c). Similar findings were reported by Savi et al. (2015b).

Although O_3 gas showed the highly efficient inhibition of fungi, the oxidation could still result in some negative effects on food quality. To the cereal itself, Savi et al. (2015c) reported that wheat could still germinate normally after 60 µmoL/moL of O_3 gas treated for 120 min. However, seed germination of wheat, maize and paddy rice was significantly affected (up to 67%) when the seeds were exposed under 4.8 mg/L for 12 h (Wang et al., 2010). For the unmilled products, ozone did not show a large impact on the total phenol content, antioxidant capacity and odour,

but the colour of some grains could fade to somewhat white colour (Wang et al., 2010; Santos Alexandre et al., 2018). In contrast, for flour products, ozonation resulted in the degradation of starch in whole wheat flour decreasing viscosity and swelling capacity and increasing the pasting temperature (Alexandre et al., 2017; Alexandre et al., 2019). The ozonation process also contributed to the peroxide value, and accelerated the oxidation of unsaturated fatty acids (Alexandre et al., 2019). Plasma treatment has good potential as a strategy to control fungal growth and aflatoxin production, but more research is needed to understand undesirable effects, including potential production of toxic compounds.

2.2.3 Biological approaches

2.2.3.1 Inhibition by microorganisms and their metabolites

In nature, fungi often share habitats with plants and with other microorganisms, and may cause competition for space and nutrients. Therefore, fungal propagation would be weakened if outcompeted by other microorganisms (Cavaglieri et al., 2005; Abbas et al., 2007; Appell et al., 2009).

This natural competition phenomenon has been exploited by researchers to control fungi and their toxins through the direct use of certain antagonist microorganisms as biocontrol agents (BCAs) or the use of microbial metabolites. Biological control of mycotoxin-producing fungi has been largely covered by several reports (Bhat et al., 2010; de Medeiros et al., 2012; Mannaa and Kim, 2016; Kong, 2017; Kagot et al., 2019). It appears that biological control using microbial antagonists such as bacteria, fungi, and yeasts could be a feasible substitute to reduce the use of antifungal chemicals. Great successes in reducing aflatoxin contamination in fields of different crops by 70 to 90% have been achieved by application of atoxigenic strains of *Aspergillus*. For the biocontrol of *Fusarium* and its associated fusariotoxins, species of *Trichoderma*, *Bacillus* and atoxigenic *Fusarium* have being tested as the most promising candidates. However, questions remain about the ability of the atoxigenic fungi to produce other mycotoxins, or to potentially exchange genetic material and become aflatoxigenic. The low efficacy of many antagonists in the

field conditions, despite showing high potential in the lab, is another concern. Overall, it is suggested that integrated management approaches should be considered, involving a combination of multiple BCAs, with reduced fungicide application, in conjunction with good agricultural practices, and coupled with good postharvest management. In this section, we focus on the inhibition of fungal growth and toxin production by microbial and plant metabolites.

Fungal growth and toxin production may be affected by metabolites produced by other microorganisms. Some proteins and peptides inhibit the growth of microorganisms and are therefore termed as antifungal peptides (AFPs) and antimicrobial peptides (AMPs). AFPs from moulds show a high stability to pH and proteolysis and exhibit a broad inhibition spectrum against filamentous fungi, and thus have prospects to control hazardous moulds in fermented foods. An AFP isolated from *Penicillium chrysogenum* (PgAFP) at 4.9 μg/mL significantly reduced the growth of *A. flavus* with over 50% inhibition rate (Delgado et al., 2015).

A compilation of the antifungal peptides produced by moulds by Delgado et al. (2016) showed 16 compounds, produced by *Aspergillus*, *Penicillium*, *Fusarium*, *Monascus*, and *Neosartorya* sp., with molecular weight (MW) between 5773 and 10,000 Da. A peptide (MW 2500 Da) isolated from *Bacillus* strain B-TL2 had strong inhibitory activity against mycelial growth of *A. niger*, as well as *Bipolaris maydis*, *Alternaria brassicae*, and *Cercospora personata.* Moreover, this peptide showed thermostability, which means the peptide could keep 100% activity at 100℃ (Zhang et al., 2008). In addition, four AMPs, namely, PPD1 (FRLHF), 66-10 (FRLKFH), 77-3 (FRLKFHF) and D4E1 (FKLRAKIKVRLRAKIKL) at concentrations between 1 to 40 μg/mL reduced the aflatoxin production by *A. flavus* and *A. parasiticus* in a dose-dependent manner. At near minimum inhibitory concentrations (MIC), the AMPs inhibited aflatoxins, without hindering the growth of the fungi. An almost 99% inhibition of aflatoxins produced by *A. parasiticus* was observed. Conidiation of the fungi was also negatively influenced by the peptides (Devi and Sashidhar, 2019). A peptide purified from *Lactobacillus plantarum* with amino acidic sequence SGADTTFLTK reduced by 73% the growth of *A. parasiticus* in liquid medium after 48 h incubation (Luz et al., 2017). Similarly, three newly identified peptides from *Bacillus megaterium* (L-Asp-L-Orn (D1O), L-Asp-L-Asn (D1N) and L-Asp-L-Asp-L-Asn (D2N)) at concentrations above 0.32 mg/well significantly inhibit the growth of *A. flavus*, but without any effect on spore germination. At concentrations

ranging between 0.04 and 0.64 mg/mL, the reduction of AFB_1 by the peptides was from 70 to 80% (Chen et al., 2019).

Efforts are being made to elucidate the mechanism of inhibition of AMPs. A single peptide is often capable of more than one mode of action, depending on the target cell type, and the antifungal activities of peptides cannot be inferred from studies on their antibacterial activities. AMPs usually act via membrane permeabilization, whereas antifungal activity for these peptides is generally more complex and often involves entry of the peptide into the cell (van der Weerden et al., 2013). As evidenced by confocal microscopy and quantitative reverse transcription polymerase chain reaction (RT-PCR), the three peptides from *B. megaterium*-D1O, D1N and D2N-could spontaneously enter into the hyphae of *A. flavus* and inhibited conidiation and aflatoxin production, but did not inhibit hyphae vegetative growth and spore germination (Chen et al., 2019). A more detailed mechanism was proposed by Devi and Sashidhar (2019), which shows that the AMPs, at concentrations near MIC, induced membrane permeabilization, without inducing cellular leakage. The AMPs also show antioxidant properties that interact with oxidative stress and impair aflatoxin production. At molecular level, the AMPs were responsible of down regulation of the aflatoxin gene cluster '*aflR*' (a regulatory gene for aflatoxin biosynthesis), and the expression of downstream genes. Similarly, a decrease in the expression of manganese-superoxide dismutase has been shown to be correlated to aflatoxin synthesis, which was obtained in peptide-treated samples.

During food fermentation, some non-peptides metabolites have been shown to have antifungal activities. These compounds produced by lactic acid bacteria included organic acids, phenol compounds, hydroxy fatty acids, hydrogen peroxide and reuterin (Dalié et al., 2010). For example, acetic and phenyl lactic acids produced by *L. plantarum* CRL 778, *L. uteri* CRL 1100, and *L. brevis* CRL 772 and CRL 796 displayed antifungal activity on *A. niger* (<40%), *Penicillium* sp. (40% to70%) and *F. graminearum* (>70%) isolated from contaminated bread. The effect of organic acids depends not only on the type of acid, but also on their concentration, the type of matrix, and pH of the matrix (Gerez et al., 2009). Selected *Lactobacillus* sp. (*L. fermentum* M107 and *L. fermentum* 223) and yeasts (*Hanseniaspora opuntiae* H17 and *Saccharomyces cerevisiae* H290) were used to inhibit the growth of *A. flavus* S075, *P. citrinum* S005 and *Gibberella. moniliformis*

S003 in cocoa bean fermentation. On average, *Lactobacillus* sp. (63% and 75% respectively) showed higher inhibition ability than yeast (25% and 31%), when they were cultural individually. Glucose, fructose, and citric acid in medium were converted to mannitol, acetic acid and lactic acid by *Lactobacillus* sp., whereas the glucose and fructose were metabolized to ethanol during culture. In the co-culture of *Lactobacillus* and yeasts, *A. flavus* S075 was inhibited completely after 10 to 14 days (Romanens et al., 2019). The antifungal interaction between fungi growth/mycotoxin production and lactic acid bacteria or yeasts was summarized by Hassan et al. (2015) and Bourdichon et al. (2012). The application of AMPs and AFPs, as well as fermentation of metabolites, seems promising strategy for fungal and mycotoxin control. Further research is needed to elucidate the mechanism of action and potential negative effects of the microbes or microbial metabolites.

2.2.3.2 Inhibition by plant extracts

Higher plants can produce a number of secondary metabolites that display wide biochemical and physiological functions (Prakash et al., 2015). A volatile substance containing secondary metabolites, obtained from distillation of plants, is called an essential oil (EO). EOs have been used for antimicrobial and insecticidal applications in the pharmaceutical, cosmetic, agricultural and food industries (Bakkali et al., 2008). EOs are mainly composed of phenylpropanoids, phenolics, terpenoids, steroids, aromatic and alkaloids, whose content determine the properties of the EOs (Bakkali et al., 2008; Prakash et al., 2015) The composition of EOs is highly variable, and it depends on plant species, modes of extraction, and storage conditions. Different parts of one plant or even the same plant harvested from diverse regions or at different harvest time can vary in antifungal ability. Table. 2-2 summarizes and compares studies of inhibition of mycotoxin-producing fungi by EOs. In a number of studies, high LD_{50} values have been recorded, such as 11 mL/kg for *Ocimum gratissimum* (Prakash et al., 2011), 4 mL/kg for *Cinnamomum glaucescens* (Prakash et al., 2013), 4.5 mL/kg for *Ocimum sanctum* L. (Kumar et al., 2010), and 9 mL/kg for *Caesulia axillaris* roxb. (Mishra et al., 2012a). Besides, the EOs of cinnamon, clove, lemon grass, oregano, thyme, nutmeg, and basil are confirmed as safe in America. In European countries, EOs components carvacrol, carvone, cinnamaldehyde, citral, p-cymene, eugenol, limonene, menthol, linalool, vanillin, and thymol are registered as flavour additives in foods (Prakash et al., 2015).

Apart from EOs, some plant AFPs and AMPs have also been identified (Yun et al., 1998; Subramanyam et al., 2012; Park et al., 2017). These components comprise defensins, lectins, chitinases, glucanases and other proteins obtained from seeds, bulbs, leaves, tubers, fruits, shoots, and roots (Yan et al., 2015). Both low-MW proteins and high-MW proteins could show the fungal inhibition capability. For example, a 5.4-kDa highly homologous plant defensins peptide purified from *Phaseolus vulgaris* L. impeded the growth of *F. oxysporum* around paper discs containing this peptide (Chan and Ng, 2013); in the same way, a designated Chitinase A (Chit A) and Chitinase B (Chit B) of 28 kDa purified from maize seeds totally inhibited *F. oxysporum* (Huynh et al., 1992); a 35.7-kDa and 65-kDa lectin from seeds of *Archidendron jiringa* and *Pachira aquatic*, respectively, showed effective effect on the growth of *F. oxysporum* (Paiva et al., 2014).

Plant antifungal metabolites are not limited to EOs, AFPs and AMPs. Polyphenols, flavonoids in particular, are a group of plant secondary metabolites that play important role on fungal defence (Bouarab-Chibane et al., 2019). The butanol extract and oxime derivative of fresh peppermint (*Mentha piperita*), which is rich in flavonoid, were found to inhibit the growth of *F. moniliforme* by Ilboudo et al. (2016). Butanol extract and oxime derivative at 5 mg/mL caused about 52% and 70% inhibition respectively. Conidial germination was delayed by the butanol extract by 1 h as compared to the control group, and less than 10% of spores germinated in total. Similarly, the oxime derivative group had less than 10% germinated spores at 2 h, after which the quantity declined. In other studies, high carotenoid content in maize could lead to low fumonisins and aflatoxins production by *Fusarium* sp. (Diaz-Gomez et al., 2016) or *Aspergillus* sp. (Diaz-Gomez et al., 2016; Suwarno et al., 2019), respectively. In a review of Atanasova-Penichon et al. (2016), phenolic acids and tocopherols were mentioned as similarly active compounds.

The diversity of plant metabolites makes plants promising sources of novel antifungal agents. Some of these can be extracted from agricultural by-products, making them potentially economically interesting. However, the variation in their composition may cause inconsistency in their performance. Purified compounds or synthetic mimetics could provide more reproducible alternatives.

Table. 2-2 Examples of mycotoxin-producing fungi inhibition by plant essential oils

Fungal species	Plant species	Organ	Region	Concentration (µL/mL)	Main components	Inhibition (%)	Reference
A. flavus	*Hedychium* sp.	Leaf	USA	40000	NM	100	Rajasekaran et al. (2012)
	Ocimum gratissimum	Leaf	India	0.6	Methyl cinnamate; γ-Terpinene	91	Prakash et al. (2011)
	Origanum majorana	NM	India	3	NM	100	Prakash et al. (2012a)
	Coriandrum sativum	NM	India	2.5	NM	100	
	Hedychium spicatum	NM	India	2.5	NM	100	
	Arachis hypogaea	Seed	India	1	NM	82	Prakash et al. (2012b)
	Arachis hypogaea	Leaf	India	1	NM	62.5	
	Cinnamomum glaucescens	Berry	India	1	1,8-Cineole	58	Prakash et al. (2013)
	Salvia officinalis	Aerial parts	Jordan	5	1,8-Cineole	100	Abu-Darwish et al. (2013)
	Artemisia herba-alba	Aerial parts	Jordan	5000	Predominant; α-and β-Thujones	100	Abu-Darwish et al. (2015)
	Caesulia axillaris	Aerial parts	India	1	$_{DL}$-Limonene; Euasarone	100	Mishra et al. (2012a)
	Jamrosa	Leaf	India	0.4	Z-citral; Linalyl acetate	100	Mishra et al. (2012b)
	Lippia rugosa	Leaf	Cameroon	1000	Geraniol; Nerol; Geranial	100	Tatsadjieu et al. (2009)
	Coleus aromaticus	Leaf	India	1	Z-citral; PrecoceneI	100	Jaya et al. (2011)
	Hyptis suaveolens	Leaf	India	1	PrecoceneI	93.8	
	Ageratum conyzoides	Leaf	India	1	Germacrene-D; Trans-caryophyllene	100	
	Ageratum conyzoides	Leaf	Brazil	1	PrecoceneI; PrecoceneII	63	Nogueira et al. (2010)
	Lavandula multifida	Aerial parts	Portugal	0.64	Carvacrol; *cis-β*-Ocimene	100	Zuzarte et al. (2012)
	Citrus sinensis var. Valencia	Orange peel	Mexico	16000	NM	100	Velázquez-Nuñez et al. (2013)
A. niger	*Ocimum gratissimum*	Leaf	India	0.6	Methyl cinnamate; γ-Terpinene	85	Prakash et al. (2011)
	Origanum majorana	NM	India	3.5	NM	100	Prakash et al. (2012a)
	Coriandrum sativum	NM	India	3	NM	100	
	Hedychium spicatum	NM	India	2.5	NM	100	
	Commiphora myrrha	NM	India	3.5	NM	100	
	Cananga odorata	NM	India	2	NM	100	
	Cinnamomum glaucescens	Berry	India	1	1,8-Cineole	63	Prakash et al. (2013)
	Salvia officinalis	Aerial parts	Jordan	5	1,8-Cineole	100	Abu-Darwish et al. (2013)
	Artemisia herba-alba	Aerial parts	Jordan	1250	Predominant; α-and β-Thujones	100	Abu-Darwish et al. (2015)
	Caesulia axillaris	Aerial parts	India	1	$_{DL}$-Limonene; Euasarone	100	Mishra et al. (2012a)
	Lavandula multifida	Aerial parts	Portugal	0.32	Carvacrol; *cis-β*-Ocimene	100	Zuzarte et al. (2012)
A. fumigatus	*Cinnamomum glaucescens*	Berry	India	1	1,8-Cineole	70	Prakash et al. (2013)
	Salvia officinalis	Aerial parts	Jordan	5	1,8-Cineole	100	Abu-Darwish et al. (2013)
	Artemisia herba-alba	Aerial parts	Jordan	2500	Predominant; α-and β-Thujones	100	Abu-Darwish et al. (2015)
	Caesulia axillaris	Aerial parts	India	1.25	$_{DL}$-Limonene; Euasarone	100	Mishra et al. (2012a)
	Lavandula multifida	Aerial parts	Portugal	0.32	Carvacrol; *cis-β*-Ocimene	100	Zuzarte et al. (2012)

A. terreus	*Caesulia axillaris*	Aerial parts	India	1	DL-Limonene; Euasarone	100	Mishra et al. (2012a)
F. verticillioides	*Hedychium* sp.	Leaf	USA	40000	NM	100	Rajasekaran et al. (2012)
F. nivale	*Ocimum gratissimum*	Leaf	India	0.6	Methyl cinnamate; γ-Terpinene	100	Prakash et al. (2011)
	Origanum majorana	NM	India	2.75	NM	100	Prakash et al. (2012a)
	Coriandrum sativum	NM	India	2	NM	100	
	Hedychium spicatum	NM	India	2.25	NM	100	
	Commiphora myrrha	NM	India	2.5	NM	100	
	Cananga odorata	NM	India	1.5	NM	100	
F. oxysporum	*Caesulia axillaris*	Aerial parts	India	0.75	DL-Limonene; Euasarone	100	Mishra et al. (2012a)
P. italicum	*Ocimum gratissimum*	Leaf	India	0.6	Methyl cinnamate; γ-terpinene	100	Prakash et al. (2011)
	Origanum majorana	NM	India	2.5	NM	100	Prakash et al. (2012a)
	Coriandrum sativum	NM	India	2.25	NM	100	
	Hedychium spicatum	NM	India	2.5	NM	100	
	Commiphora myrrha	NM	India	2.5	NM	100	
	Cananga odorata	NM	India	1.5	NM	100	
	Caesulia axillaris	Aerial parts	India	1	DL-Limonene; Euasarone	100	Mishra et al. (2012a)

NM: Not mentioned

2.2.4 Combined approaches

Most approaches have limitations in terms of specificity to fungi and food matrices. For this reason, combinations of approaches have been tested by researchers, to offer an integrated management strategy that can target multiple microorganisms in various matrices.

The combination of MAP and antifungal additives can prolong the shelf life of life. For instance, pre-treatment with 3% potassium sorbate and 20% of ethanol solution decreased the incidence of moulds and yeasts on table grapes by approximately 11 to 30% higher than those only packed in MAP conditions (O_2:CO_2:N_2 [6:5:89]) at 4°C until the end of shelf life. Meanwhile, 3000 ppm of citrus extract only caused a 9% decrease in the same system (Cristina et al., 2013). Similarly, the population of yeasts and moulds on ready-to-cook poultry product in 1.5% chitosan and 0.2% thyme extract under MAP conditions (30% CO_2 and 70% N_2) was 2.2 log CFU/g lower than that under the MAP only when stored at 4 ◦C for 14 days. The antifungal effect of chitosan and thyme was greater when used in combination compared to each individually (Giatrakou et al., 2010).

Yoon et al. (2014) and Jeong et al. (2016) used irradiation in combination with the chemical sodium dichloroisocyanurate (NaDCC) and to reduce the activity of grey mould (*Botrytis cinerea*) and green mould (*P. digitatum*). Their results indicated that the decrease of quantity of grey mould relied on the increase of radiation (from 0.2 to 4 kGy) and increase in NaDCC (from 5 to 50 ppm) dose, but the great reduction (<5%) of green mould only occurred under the treatment of 0.4 kGy of gamma irradiation and 6 or 10 ppm of the NaDCC. Combining physical with chemical approaches appears to be effective at preventing fungal growth. More research is needed to understand the combined effects on a wider range of microbes and food matrices.

2.3 Reduction of mycotoxin content

The scale of food production makes control of fungal growth challenging. It is not always possible to prevent fungal growth or mycotoxin contamination. Therefore, approaches to remove mycotoxins from the edible part of the food must be considered.

2.3.1 Physical detoxification approaches

2.3.1.1 Cleaning, dehulling and milling

Sorting and cleaning are the most common and cost-effective mycotoxin removal processes. Matumba et al. (2015) investigated the effect of sorting, flotation/washing, or dehulling on the levels of 11 mycotoxins in white maize grown in Malawi. In general, hand sorting showed the greatest reduction of mycotoxins (more than 90%), followed by dehulling (more than 70%, except DON and AcDON). When the procedures were combined, less than 5% of mycotoxins could be detected. Similarly, it is reported that the level of aflatoxins, fumonisins, DON, NIV, and ZEN in washed food samples was lower than in original samples (van der Westhuizen et al., 2011; Tibola et al., 2016; Matumba et al., 2017). According to Tibola et al. (2016), the lowest mycotoxin level could be obtained in milled flour products. Notably, the milling could cause a redistribution of mycotoxins in milling fractions. In general, lower mycotoxin contents are found in flour and semolina, whereas the higher mycotoxins content is found in brans and flour shorts screenings. These fractions were generally used for animal feeding (Cheli et al., 2013). An educational intervention trained women in Gambia to recognize and remove mouldy groundnuts by hand sorting. The intervention resulted in a reduction of 42.9% AFB_1 (based on median AFB_1 levels at baseline and after hand sorting), and a reduction of 96.7% (based on the total AFB_1 in mouldy and clean groundnuts), with a loss of only 2% of the groundnuts. By roasting the already clean sorted groundnuts, AFB_1 reduction was 39.3% achieved (based on median levels) (Xu et al., 2017). Due to the low cost and easy operation of sorting and cleaning, these procedures can be used not only before crop storage, but also during other processing operations before food consumption. However, it is still necessary to consider the disposal of sorted contaminated seeds and wastewater containing mycotoxin.

2.3.1.2 Heat treatment

The majority of mycotoxins are heat stable. Aflatoxins and OTA could be partially destroyed at the temperatures around 250 and 200℃ respectively. The complete degradation of fumonisins takes place at over 180℃ (Magan and Olsen, 2004; Vidal et al., 2015), and for DON degradation takes place at 210℃ (Milani and Maleki, 2014). In general, mycotoxin destruction is dependent

on both the temperature and duration of exposure. Degradation of AFB_1 and AFB_2 in pistachio nuts was proportional to both temperature (90 to 150℃) and treatment time (30 to 120 min) during roasting processing, although this degradation was more affected by temperature than by time (Yazdanpanah et al., 2005). In ground corn, Dupuy et al. (1993) found a linear relationship between calculated half-lives of FB_1 and temperature, which were 8 h, 175 min, 38 min, and 10 min at 75, 100, 125 and 150℃, respectively.

For more efficiency reducing of mycotoxins during food processing, high temperature can be combined with high pressure. Extrusion cooking is a food procedure that uses high temperature and high pressure to process foods in a short time, which usually applied in relatively dry viscous material (MCs around 20%), such as cereal grains, grits, and flours (Castells et al., 2006). In general, initial moisture content of food materials, extruded temperature, processing duration, screw speed and mycotoxins type are the main variables influencing the reduced efficiency of mycotoxins. In rice meal, aflatoxins were reduced by 51 to 95% during the extrusion cooking. Broadly speaking, the longer the processing duration, the higher the reduction of aflatoxins content. In rice, 170℃ was the best temperature among three temperatures (140, 170, 200℃) for reducing AFB_1 and AFB_2, and no significant difference on AFG_1 reduction between 170℃ and 200℃, while AFG_2 was most reduced at 200℃ (Castells et al., 2006). For OTA in oat flakes, according to a central composite design analysis, the highest reduction of 28% could be obtained at 162℃, 30% moisture and 221 rpm of screw speed (Lee et al., 2017). Pleadin et al. (2019) compared the effects of different thermal treatment on reduction of DON and ZEN in cereals. The results showed the content of DON and ZEN only reduced by 11% in 30 min by cooking at 96°C, whereas the DON and ZEN declined up to 40% and 46% when the roast temperature was increased to 220°C. The highest degradation of DON and ZEN was 75% and 80%, respectively, by extrusion cooking (135°C to 190°C).

2.3.1.3 Irradiation treatment

The ionizing radiation can not only inhibit growth and development of fungi, but also reduce some mycotoxins, and the effect is dose dependent. In general, there is a positive correlation between irradiation dose and reduction effect in same matrix (Herzallah et al., 2008; Jalili et al.,

2012; Kumar et al., 2012). Meanwhile, irradiation also shows diverse performance on mycotoxin detoxification between irradiation types. Kumar et al. (2012) obtained 93% of OTA reduction in aqueous coffee bean by gamma irradiation at 5 kGy. Herzallah et al. (2008) achieved the destruction of about 30% of total aflatoxin and AFB_1 at 2450 MHz and 1.45 kW for 10 min. Sunlight (solar irradiation) reduced more than 60% aflatoxins under 30 h exposure and about 40% aflatoxins under 3 h exposure (Herzallah et al., 2008). Recently, electron beam irradiation has been used for decontamination of ZEN and OTA in maize kernel and maize flour. At the dose of 50 kGy, the degradation of ZEN was approximately 60% and 71% for maize flour and maize kernel respectively, and those of OTA were about 60% and 73% respectively (Luo et al., 2017). PAT was successfully reduced using UV radiation. In a study on PAT degradation in apple juice or apple cider using ultraviolet C (UVC) wavelengths of 200 to 280 nm, Zhu et al. (2014) found that the average UV fluences of 19.6, 84.3, 55.0, and 36.6 mJ/cm^2 resulted in 90% reduction of the toxin, with the order of efficiency of the three wavelength lamps was as following: far UVC (222 nm) > far UVC plus (282 nm) > UVC (254 nm). A nonsignificant increase in the *L** (lightness) value and decreases in *a** (redness) and *b** (yellowness) values of the juices treated with 222 nm were obtained. The treatment also resulted in 36.5% loss of juice ascorbic acid. Assatarakul et al. (2012) reported a reduction of PAT from 72.57 to 5.14% with UV exposure, ranging from 14.2 mJ/cm^2 (one pass) to 99.4 mJ/cm^2, respectively, from an initial PAT contamination of 1000 ppb. The UV treatment did not significantly change titratable acidity and ascorbic acid of the juice, but there was modification of the pH, the degrees Brix and in sensory perception for the finished apple juice. In a similar study, Kim et al. (2018) observed that in PAT-spiked apple juice samples were UV-irradiated at a range of 200 to 280 nm for different time intervals, PAT levels reduced from 94.11 μg/L to 69.28, 54.55, and 5.92 μg/L after 5, 10, and 30 min, respectively. After 30 min of UV exposure, PAT was not detected in spiked apple juice samples. However, UV irradiation reduced the yellowness (*b*∗) of apple juice.

The matrix is another factor to reflect different detoxification efficiency. With gamma radiation dose of 10 kGy, OTA in methanolic suspension demonstrated 24% lower reduction than same concentration of the toxin in water. OTA powder the lowest reduction effect by gamma radiation (Kumar et al., 2012). At gamma irradiation dose of 1 kGy, compared to practical degradation in

distilled water, the degradation rate of PAT in 1% organic acid solutions (malic acid, citric acid, lactic acid, acetic acid), 1% amino acid solutions (aspartic acid, serine, threonine and glutamic acid, histidine), ascorbic acid and ethanol ranged from 31 to 98%. Therefore, in irradiated apple juice, 33% of PAT retention was due to its main elements of organic acid (5.68% of malic acid) and amino acid (0.08% of serine and 0.06% of threonine) (Yun et al., 2008). In another study, however, the detoxification of ZEN between distilled water and all orange juice, pineapple juice and tomato juice had no significant difference. However, the optimized model analysed by response surface methodology concluded that the determinant factors of detoxification were both irradiation dose and ZEN concentration in fruits juices. It was noted that irradiation-mediated detoxified ZEN showed lower toxicity than nonirradiated ZEN in cell line models. Furthermore, to assess the quality of fruit juices, the sensory profile, total phenolic content, total flavonoid content, total antioxidant activity and acidity were taken into account. In three fruit juices, the values of every parameter slightly decreased with increasing irradiation dose of 2.5, 5 and 7.5 kGy, while 10 kGy of irradiation had significant deterioration on quality parameters. Overall, irradiation with certain dose range could be used to reduce toxin content of fruits juices (Kalagatur et al., 2018).

When the irradiation is applied on foods, the primary reaction is the ionization of water, which decomposes the water molecules into positively charged water radicals and negatively charged free solvated electrons. Next, the water radical is split into hydroxyl radicals and hydrogen ions. The reaction ends until forming the final products of hydrated electrons, hydroxyl radicals, hydrogen ions, and hydrogen atoms. The radicals can be added into double bounds of mycotoxins, such as aromatic rings, heterocyclic rings and lactone rings, which lowers the mutagenicity and toxicity of mycotoxins (Jalili et al., 2012; Di Stefano et al., 2014). Irradiation looks a promising approach to reduce mycotoxins content in fruit juices. Other matrixes, including dried products, need to be considered as well. Besides, the mycotoxin degradation products need to be assessed for their toxicity. Further development to prevent quality deterioration as a result of irradiation is necessary.

2.3.2 Chemical detoxification approaches

2.3.2.1 Adsorption by chemical adsorbents

Some chemicals form weak interactions with mycotoxins due to their characteristics including polarity, solubility, molecular size, shape, and surface area and, in the case of ionized compounds, charge distribution and dissociation constants (Sabater-Vilar et al., 2007; Jard et al., 2011; Sun et al., 2018), causing adsorption between adsorbents and mycotoxins. Hydrated sodium calcium aluminosilicates (HSCASs) are one of the most popular clay-based adsorbents, obtained from natural zeolite (Şişman, 2006). The adsorption of pyrophyllite-type HSCAS, usually occurs in either or both octahedral and tetrahedral layers (the structure can be found in El Gaidoumi et al. (2019) report) causing weak bonds of exchangeable cations in interlayer positions (Aly et al., 2004). Apart from HSCAS, many other adsorbents, displaying diverse adsorption ability, are shown in Table 2-3. These adsorbents included clay, activated charcoal, esterified glucomannan, cholestyramine, and other modified polymers, showing 17 to 100% adsorption of AFB_1, FB_1, DON, ZEN, OTA, and T-2 in liquid environments. There is no doubt that adsorbent adsorption is one of the most economical methods in mycotoxin reduction. Nevertheless, the safety of the absorbent materials, removal from foods, and disposal of adsorption chemicals and adsorbent mycotoxin complex are still under question. Some chemical adsorbents have been forbidden as detoxification materials in food industry by the European Union (Jard et al., 2011).

Chemical adsorbents find more practical applications in mycotoxins detoxification of animal feeds. As a source of animal protein (milk, meat, and eggs), the contaminated livestock products can result in direct or indirect risk to human health (Halász et al., 2009). For example, AFB_1 can be converted into aflatoxin M_1 (AFM_1) in cattle bodies, which is secreted in milk and consumed by humans, especially children (Peng et al., 2018). Adding additives into fodder is a low-cost and user-friendly detoxification method in animal feeding, and has been used in practical production. These additives mainly include aluminosilicate clays (with or without organic acid) and montmorillonite (Table 2-3), which are not always appropriate for human foods (Kolosova and Stroka, 2012). Although some in vivo studies have shown that the feeding additives decreased the impact of mycotoxins on growth and did not increase the toxicity in animals, adding additives

Table. 2-3 Mycotoxins detoxification by chemical adsorption in different matrixes

Sorbents	Mycotoxin	Concentration (mg/mL)/(mg/mg)	Effects	Time	Matrix	Reference
Hydrated sodium calcium aluminosilicates (HSCASs)	AFB_1	40	>97% Adsorption	30 min	Malt suspension	Aly et al. (2004)
		0.01	Retard of the decline in the total number of offspring	30 d	*In vivo* (*Drosophila melanogaster*)	Şişman (2006)
		2	No significant decrease on body weight gain; Retention on crude protein; Decrease on crude fat;	42 d	Broilers basal maize-soybean meal	Liu et al. (2011)
		3	No significant decrease on calcium; Retention on the proportion of breast muscle, thigh muscle and abdominal fat; Improvement of growth performance, digestibility, and immune function; Reduction of deleterious effects and tissue residues caused by AFB_1	21 d	Broilers maize meal	Liu et al. (2018)
	FB_1	40	>84% Adsorption	30 min	Malt suspension	Aly et al. (2004)
	OTA	2	No significant decrease on body weight gain; Retention on crude protein; Decrease on crude fat; No significant decrease on calcium; Retention on the proportion of breast muscle, thigh muscle and abdominal fat	42 d	Broilers basal maize-soybean meal	Liu et al. (2011)
	ZEN	10	50% Adsorption	90 min	Acetate/or citrate buffer	Yiannikouris et al. (2013)
		5	Reestablishment of haematological parameters, levels of serum biochemical enzyme activities and histological pictures of both liver and kidney	48 h	*In vivo* (mice)	Abbès et al. (2006)
	T-2	2	No significant decrease on body weight gain; Retention on crude protein; Decrease on crude fat	42 d	Broilers basal maize-soybean meal	Liu et al. (2011)
Hydrated sodium aluminosilicate	AFB_1	5	No effect on hepatic lesions	1 year	Rainbow trout diet meal	Arana et al. (2011)
Activated charcoal	FB_1	2	100% Adsorption	1 h	Aqueous solution	Galvano et al. (1997)
	OTA	0.4	>95% Adsorption	1 h	Aqueous solution	Galvano et al. (1998)
	DON	2	>90% Adsorption	1 h	Aqueous solution	Galvano et al. (1998)
		1	67% Adsorption	90 min	Phosphate buffer	Cavret et al. (2010)
	ZEN	1	100% Adsorption	90 min	Phosphate buffer	Cavret et al. (2010)
Clay	AFB_1	0.002	Reduction of effects by mycotoxins on immune system and the liver; Improve pig growth	42 d	Pig meal	Weaver et al. (2013)

	OTA	0.002	Reduction of effects by mycotoxins on immune system and the liver; Improve pig growth	42 d	Pig meal	Weaver et al. (2013)
Egyptian montmorillonite	AFB_1	40	>97% Adsorption	30 min	Malt suspension	Aly et al. (2004)
	FB_1	40	>80% Adsorption	30 min	Malt suspension	Aly et al. (2004)
Esterified glucomannan	AFB_1	0.5	No significant decrease on body weight gain; Increase on crude protein; No significant decrease on calcium; Retention on the proportion of breast muscle, thigh muscle and abdominal fat	42 d	Broilers maize-soybean meal	Liu et al. (2011)
	OTA	0.5	No significant decrease on body weight gain; Increase on crude protein; No significant decrease on calcium; Retention on the proportion of breast muscle, thigh muscle and abdominal fat	42 d	Broilers maize-soybean meal	Liu et al. (2011)
	T-2	0.5	No significant decrease on body weight gain; Increase on crude protein; No significant decrease on calcium; Retention on the proportion of breast muscle, thigh muscle and abdominal fat	42 d	Broilers maize-soybean meal	Liu et al. (2011)
Cholestyramine	DON	0.82	10% Adsorption	4 h	Phosphate-citrate buffer	Döll et al. (2004)
		1	65% Adsorption	90 min	Phosphate buffer	Cavret et al. (2010)
	ZEN	0.82	94% Adsorption	4 h	Phosphate-citrate buffer	Döll et al. (2004)
Modified aluminosilicate	DON	0.82	17% Adsorption	4 h	Phosphate-citrate buffer	Döll et al. (2004)
	ZEN	0.82	81% Adsorption	4 h	Phosphate-citrate buffer	Döll et al. (2004)
PVP-DEGMA-TAIC	FB_1	0.005	86% Adsorption	24 h	Wine-like model solution	Carrasco-Sanchez et al. (2017)
	FB_2	0.005	94% Adsorption	24 h	Wine-like model solution	Carrasco-Sanchez et al. (2017)
Poly(acrylamide-co-ethyleneglycol-methacrylate)	FB_1	0.005	82% Adsorption	24 h	Wine-like model solution	Carrasco-Sanchez et al. (2017)
	FB_2	0.005	100% Adsorption	24 h	Wine-like model solution	Carrasco-Sanchez et al. (2017)
Trimethylstearyl ammonium bromide	AFB_1	5.8	89% Adsorption	1 h	Phosphate buffer	Sun et al. (2018)
	ZEN	5.9	86% Adsorption	1 h	Phosphate buffer	Sun et al. (2018)

PVP-DEGMA-TAIC: Resins of copolymerization of N-vinyl-2-pyrrolidinone with ethylene glycol dimethacrylate and triallyl isocyanurate

could still cause the loss of essential nutrients and decline in growth performance to some extent. Thus, additives are not recommended for extensive use (Kolosova and Stroka, 2012).

2.3.2.2 Alkaline/acid treatment

Many major toxins are unstable in alkaline environments. Researchers have worked on the effect of common alkaline reagents on mycotoxin reduction. $Ca(OH)_2$, NaOH, KOH and $NaCO_3$ were found to reduce of DON, ZEN, aflatoxins, OTA (Jalili et al., 2011). In alkali treatment, ammoniation is one of the best documented studied methods of reducing toxins. So far, it has been demonstrated that ammonia could reduce almost all of aflatoxins (Brekke et al., 1977), including 45% of FB_1 (Norred et al., 1991), and 64% of ZEN (Bennett et al., 1980). This treatment was more widely used in animal feeding from last century. In the 1970s, 1.5% ammonium hydroxide was added into aflatoxin-contaminated maize basal diet of rainbow trout, which caused the detoxification of aflatoxin in diet and decrease of hepatocarcinogenicity in rainbow trout (Brekke et al., 1977). Later, in the 1990s, Bailey et al. (1994) reported that ammoniated aflatoxin-contaminated cottonseed, a kind of cattle feedstock, led to a 94% reduction in the content of AFB_1. When the rainbow trout (*Oncorhynchus mykiss*) ate the dried milk from the cattle fed by treated cottonseed meals, the incidence of hepatic tumors decreased by around 40%. Into the 21^{st} century, ammonia vapor was used in decontamination of broiler chick diet. Broilers fed diets containing aflatoxin showed the high mortality rate (about 30% in 6 weeks) during the rearing period. Chicks fed ammonia-treated maize did not show significant differences on mortality rate, dietary intake, body weight gain, and feed conversion ratio of chicks (Allameh et al., 2005). Ammonia treatment did not significantly affect the detoxification of FB_1 in maize meal under air condition (Norred et al., 1991). This may be because ammonia could directly attack the lactone ring of aflatoxins and retain the difuran moiety, but had no direct reaction sites in FB_1 (Norred et al., 1991; Karlovsky et al., 2016; Temba et al., 2016b). Furthermore, DON (Fig. 2-1) has been found to be mainly degraded to norDON A, norDON B, and norDON C (Fig. 2-2a-c) in alkaline environments. These degraded compounds could be isolated from NaOH solution (75°C, 60 min) and other processed samples, and have been shown be less toxic than original DON. Other 4 new compounds, norDON D, norDON E, norDON F and 9-hydroxymethyl DON lactone (Fig. 2-2d-g), were identified as degradation compounds (Bretz et al., 2006). In many reports, alkaline

ammonia treatment was mainly reported in the 1990s, and primarily used in animal feeding. This might be because the safety and applicability of alkaline ammonia treatment could not completely used in the food industry. However, in recent reports, baking soda has been shown to reduce the content of OTA in cereal-based foods. In an 85°C direct steam injected process that exposes food to high temperature with high steam pressure, 19.8% of OTA in oat-based infant cereals was lost. In contrast, OTA reduced by 36.1% and 43.4% when 0.5% and 1% baking soda was added, respectively (Lee et al., 2019). Peng reported that a small decline of OTA (6.73 to 9.63%) occurred in Chinese fried bread sticks containing 0.4% soda during processing (Peng et al., 2015).

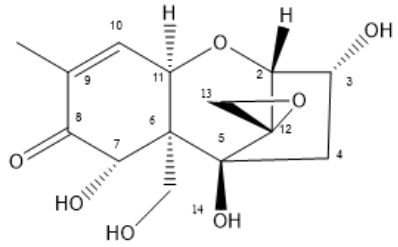

Fig. 2-1 Structure formula of DON (Wu et al., 2017)

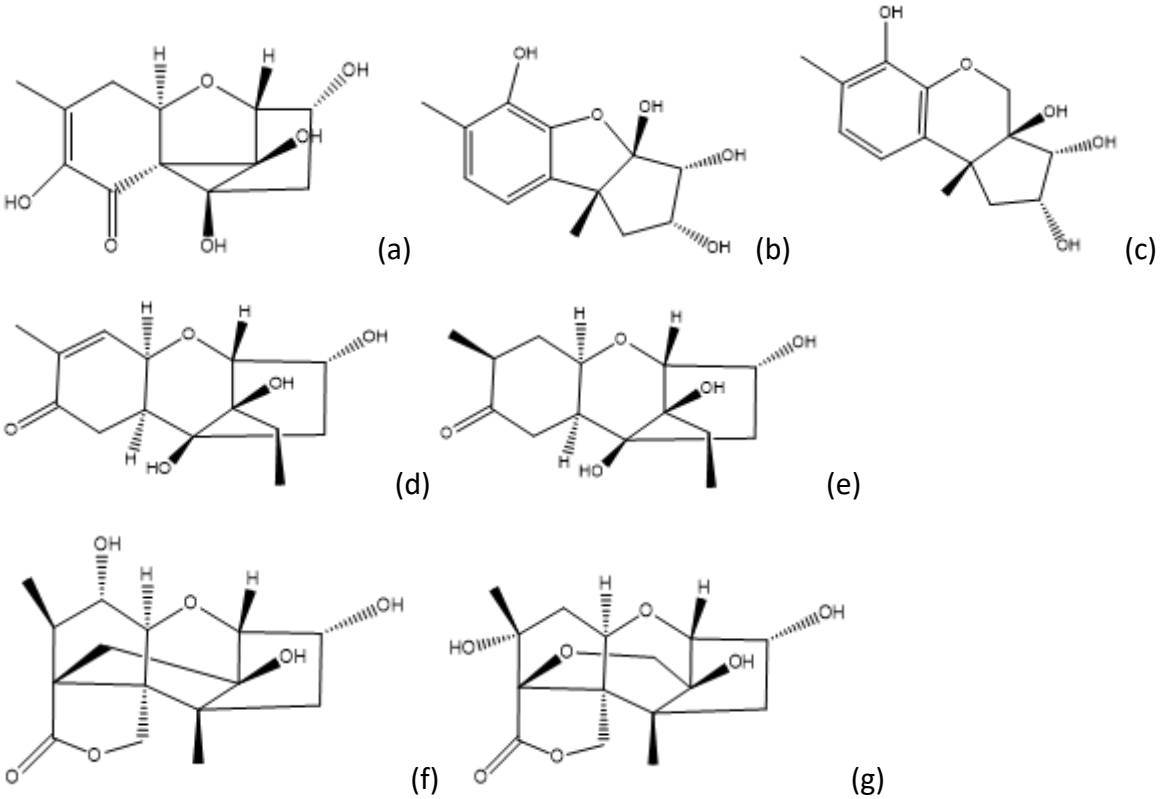

Fig. 2-2 Structure formula of norDON A (a), norDON B (b), norDON C (c), norDON D (d), norDON E (e), norDON F (f), and 9-hydroxymethyl DON lactone (g) (Bretz et al., 2006)

Although the majority of mycotoxins are resistant to weak acids (Karlovsky et al., 2016), some acids also influence the presence of mycotoxins. Sulfuric acid, chloridric acid, phosphoric acid, benzoic acid, citric acid and acetic acid all displayed less than 30% reduction of AFB_1, AFB_2, AFG_1, AFG_2 and OTA in black and white pepper during washing, which was generally less effective than that in alkaline solutions (Jalili et al., 2011). In another study, these five toxins were treated by 2% sodium hydrosulphite ($Na_2S_2O_4$) with atmospheric pressure (low pressure) and 100℃ for 30 min or 1.5 bar (high pressure) and 121℃ for 15 min. Except for AFB_2, other four samples under low pressure lost toxins of 64.8 to 83%, and those under high pressure lost more than 96% (Jalili and Jinap, 2012).The use of 5% of both citric acid and lactic acid reduced DON about 20 to 40% in feeds soaked for more than 5 h. Lactic acid showed the better performance than citric acid in this treatment (Humer et al., 2016). The detoxification of DON is considered to be due to the opening of the C12, 13-epoxy group (Fig. 2-1). In the extreme acidic environment (pH 1 to 2), DOM-1 (Fig. 2-3) might be degraded from DON (Wu et al., 2017).

Sometimes, a combination of chemicals can reduce the level of mycotoxins. In the report of Rempe et al. (2013), a mixture of methylamine, $Ca(OH)_2$ and sodium metabisulphite (2+4+1) caused the recession of 91% of DON and 79% of ZEN in naturally contaminated maize at 80°C. However, there is little evidence to show the safety of new derivatives produced from mycotoxin after treatment.

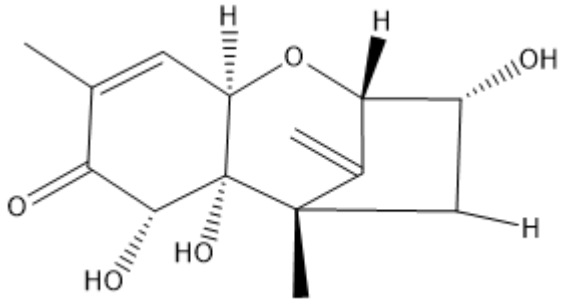

Fig. 2-3 Structure formula of DOM-1 (Wu et al., 2017)

2.3.2.3 Plasma treatment

Plasma is an ionized gas, with zero net electrical charge, that can be induced in any neutral gas, and able to induce by innovative physical equipment at pressure and temperatures conditions (Misra et al., 2019). Many studies have suggested the mycotoxin detoxification effect of plasma. For instance, the effect of cold atmospheric plasma on aflatoxin contamination in both solution matrix (liquid) and hazelnuts matrix (solid) were evaluated by Siciliano et al. (2016). In this study, gas composition (proportion of N_2 and O_2), power of generator, exposure time and reaction matrix were factors tested on detoxification efficiency. Among them, the power and exposure time were inversely proportional to aflatoxins loss. Besides, gas mixture with more N_2 and liquid matrixes were more conducing to aflatoxin degradation. When AFB_1, DON and NIV were exposed to self-designed microwave-induced argon plasma system, the decrease of these toxins was significantly time dependent, with complete in 5s (Park et al., 2007).

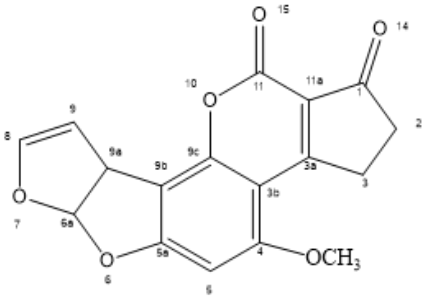

Fig. 2-4 Structure formula of AFB_1 (Luo et al., 2014)

Ozone was also used in the degradation of mycotoxins. For instance, highest reduction (48% and 64.3% respectively) of total aflatoxins and DON in soft wheat grains occurred at the 60 mg/L of concentration for 300 min (Trombete et al., 2017). ZEN in whole wheat powder quickly reduced 62.3% in first 20 min at the condition of 51 mg/L of ozone (Alexandre et al., 2019). Fifteen-minute treatment by ozone on wheat bran contaminated with DON and ZEN caused approximately 29% and 52% degradation, respectively, and there was no significant difference in the longer treatment time. Notably, ozonisation protected the quality of wheat bran by keeping total phenolic compound content and antioxidant activity (Santos Alexandre et al., 2018). The effectiveness not only depends on exposure time and gas concentration, but also the physical characteristic of samples, MC and processing method (Trombete et al., 2017). Wang et al. (2016)

showed that ozone treatment at same concentration was more effective in the flour than whole wheat with increasing of ozone concentration (from 0 to 100 mg/L), suggesting that penetration into the kernels is not effective. Meanwhile, in the same study, higher MC (20.1%) of both whole wheat flour and wheat kernels showed greater degradation of DON (about 75% and 60%, respectively), as high MC might promote oxidation power of ozone and its penetration ability. Similar result could be found in Alexandre et al. (2017) study as well. Wang et al. (2016) compared the degradation of AFB_1 by a dry method that involved delivering the O_3 gas to cereals, compared to an aqueous method that involved soaking cereals into ozone solutions and a semi-wet method that involved pumping ozone-rich steam into cereals. The results indicated that the ozonation reaction in aqueous or semi-wet conditions showed better effect than the dry method. The most effective reduction method for paddy rice and maize was semi-wet method, which reduced toxin content by about 92% and 85%, respectively, whereas the aqueous method displayed the best performance on AFB_1 degradation (about 93%) in wheat.

Ozone preferentially attacks the unsaturated compounds in an electrophilic attack mechanism (Freitas-Silva and Venancio, 2010). The major mycotoxins including aflatoxins, FB_1, OTA, ZEN, DON and PAT could be degraded rapidly, within minutes. After ozone treatment, none of the by-products of OTA, ZEN and PAT could be detected by UV or fluorescence detector. However, a larger fraction of polar compounds was formed from ozonized AFB_1, and FB_1 formatted. For DON, ozone attacked at the C9 to C10 double bond (Fig. 2-1) with two additional atoms of oxygen but kept the rest of molecule (McKenzie et al., 1997; Young et al., 2006). It was mentioned in the review of Misra et al. (2019) that plasma interacts with mycotoxins via free radicals (e.g. O•, OH•) of plasma. With AFB_1 (Fig. 2-4), for example, the degradation is through epoxidation and oxidation by introducing water molecule, hydrogen atom, aldehyde group or hydroperoxyl radical (HO_2•) and leading the breakdown of C8 to C9 double bond of the dihydrofuran rings. Meanwhile, the toxicity and carcinogenicity of AFB_1 would be reduced because of the loss of terminal furan ring. In bioassay, apart from FB_1, all of treated aflatoxins, OTA, ZEN and PAT were not found to affect the activity of *Hydra Attenuate*, but treated FB_1 still kept the toxicity to it (McKenzie et al., 1997). In the induced toxicity assay of Caco-2 cells, ozone treatment weakened the cellular metabolic disorder by DON derivatives and it has no impact on latent inflammation

and oxidative stress effects, which shows some of the nonnegligible toxicity of ozonized DON (Xu et al., 2019). It is noteworthy that low O_3 concentration (below 0.05 ppm) had an enjoyable odour, whereas, when the concentration was above 0.05 ppm, O_3 affected human eyes and respiratory systems, which might be related to premature death, heart attack, bronchitis, asthma, and other cardiopulmonary problems (Jian et al., 2013). Therefore, when considering the application of ozone in cereal storage, attention should be paid to the harm caused by ozone to workers and the natural environment. Plasma, and in particular ozone, is effective at reducing mycotoxin content in foods. However, the toxicity of degradation products and impacts of ozone directly on human health need to be further considered.

2.3.2.4 Neutral EOW

Neutral EOW is also an aflatoxin detoxifying substance. One view was that aflatoxin detoxified by hypochlorous acid from EOW eliminated the toxicity of double bond in the terminal furan ring and converted it to 8-chloro-9-hydroxy-aflatoxin B_1 (Fig. 2-5) (Escobedo-González et al., 2016). It The derivative was shown to have significantly lower cytotoxicity and genotoxicity effects *in vitro* in a HepG2 cell model (Jardon-Xicotencatl et al., 2015; Sakudo et al., 2017). There was an ameliorative effect of EOW on the health and performance of turkeys fed on decontaminated feed (Gómez-Espinosa et al., 2017).

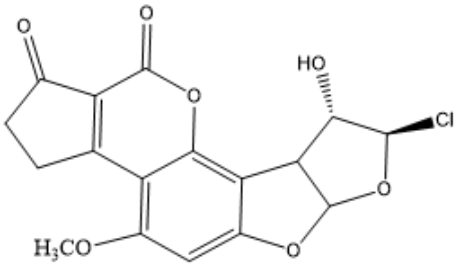

Fig. 2-5 Structure formula of 8-chloro-9-hydroxy-aflatoxin B_1 (Escobedo-González et al., 2016)

2.3.3 Biological detoxification approaches

2.3.3.1 Metabolite degradation

Biological enzymatic degradation reactions include acetylation, glucosylation, ring cleavage, hydrolysis, deamination, and decarboxylation caused by extra- or intracellular enzymes produced

from bacteria and fungi (Hathout and Aly, 2014). In a report of Guan et al. (2008), *Stenotrophomona smaltophilia* was isolated from a selective medium containing coumarin as the only carbon source and displayed reducing ability towards AFB_1 (82.5%) at 37℃ for 72 h. After treatment of factors that could affect the enzymatic activity, reaction efficiency significantly drops, which indicated that reduced AFB_1 was produced by enzymatic degradation. Microbial species with similar functions were listed by Hathout and Aly (2014), including *Bacillus* sp., *Brevibacterium* sp., *Eubacterium* sp., *Flavobacterium aurantiacum*, *Mycobacterium fluoranthenivorans*, *Myxobacteria* sp., *Pseudomonas* sp., *Rhodococcus erythropolis*, *Trichosporon mycotoxinivorans*, *Aspergillus* sp., and *Rhizopus* sp. The degraded toxins covered all major mycotoxins. Besides, some enzymes had been found in mushroom showing the detoxification ability. Manganese peroxidase purified from mushroom *Pleurotus ostreatus* detoxified AFB_1 by 6% at 0.1 U/mL enzyme activity for 8 h, and by 90% at 1.5 U/mL enzyme activity for 48 h (Sayed, 2014). In a review of Jard et al. (2011), the multiple degradation pathways of each major mycotoxins has been summarized. In simple terms, the lactone ring or difuran ring of AFB_1 (Fig. 2-4) could be opened resulting in loss of toxicity. OTA was degraded to OTα and phenylalanine (Fig. 2-6); whereas ZEN could be transformed into oxidised compounds, hydroxylated and methylated compounds, gluco- or sulfo-conjugates and hydrolysed compounds. Detoxification of DON was done by opening the 12,13-epoxy ring (Fig. 2-1), and formation de-epoxidized DON and 3-keto-DON (Fig. 2-8); FB_1 has been found to be converted into polyolamine (Fig. 2-9) by extracellular carboxylesterase. Most degradation products showed no toxicity. However, the produced α-zearalanone (classified as hydroxyl compound) was more toxic than the original compound (ZEN) (Figure 7).

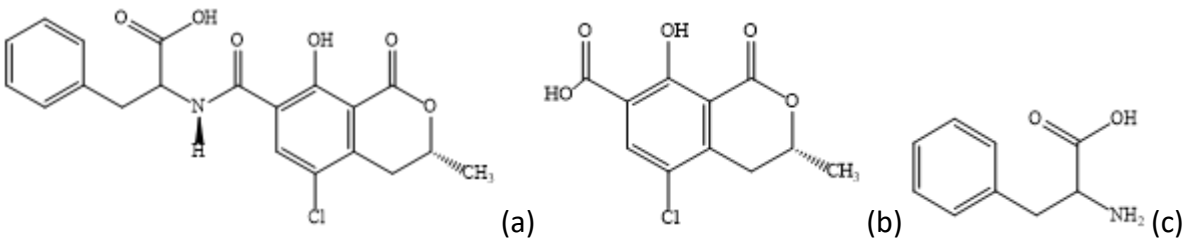

Fig. 2-6 Structure formula of OTA (a), OTα (b), and phenylalanine (c) (Jard et al., 2011)

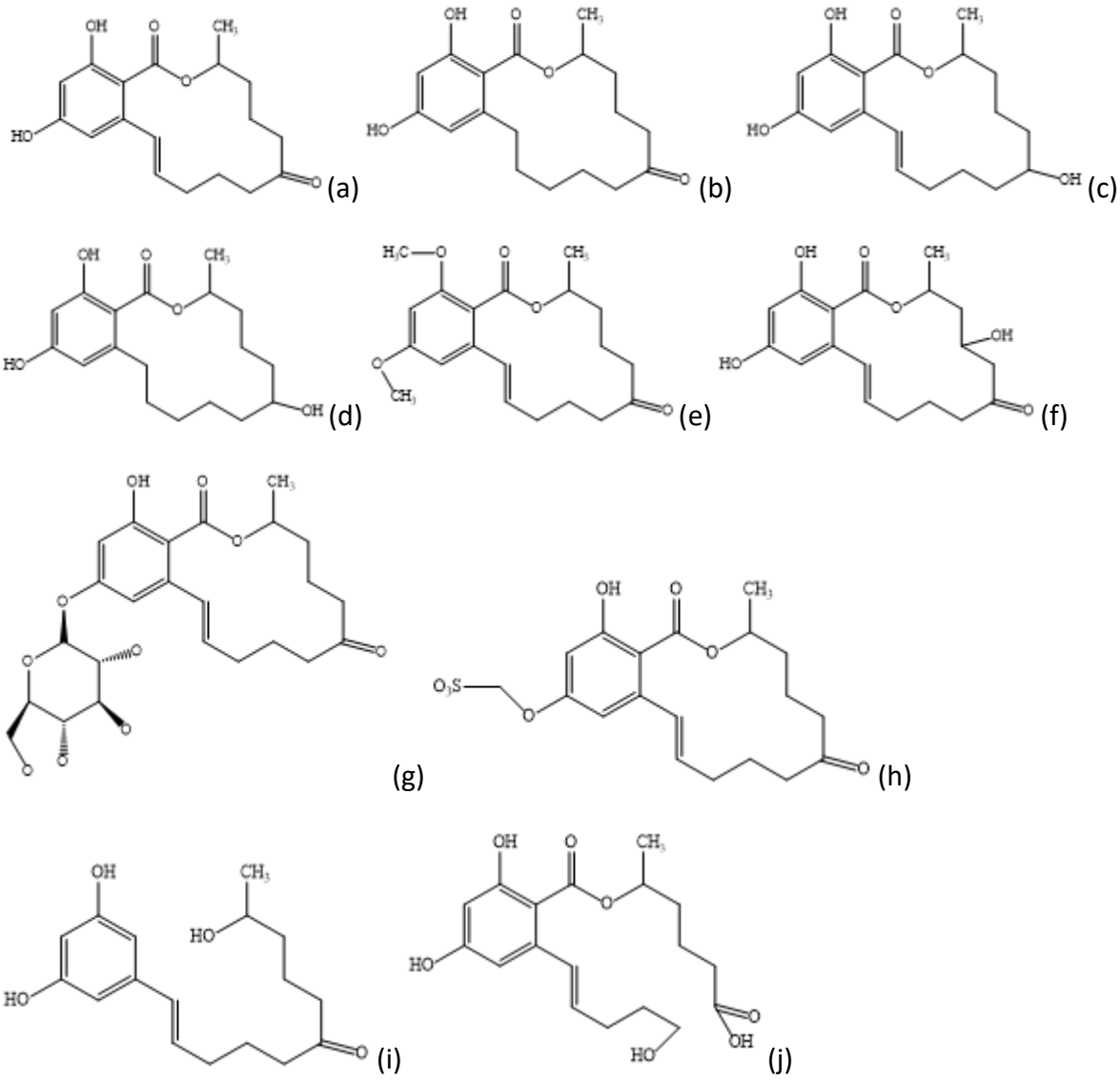

Fig. 2-7 Structure formula of ZEN (a), oxidized compounds: zearalanone (b), hydroxyled and methyl compounds: a-β zearalenol (c), a-β zearalanol (d), methoxy-ZEN (e), hydrocy-ZEN (f), gluco- or sulfo-conjugates: ZEN-4- β-glucopyranoside (g), ZEN-4-sulfate (h), and hydrolyzed compounds: decarboxylated ZEN (i), Hydroxylated ZEN (j) (Jard et al., 2011)

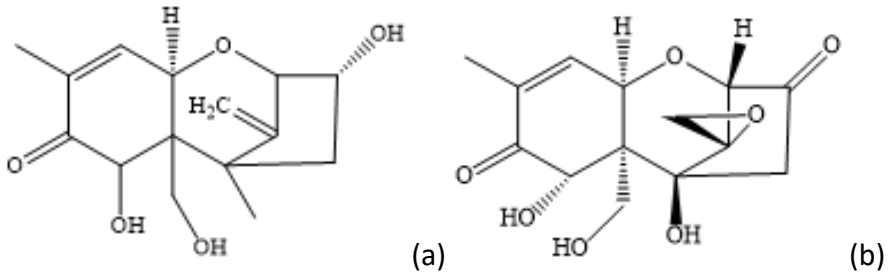

Fig. 2-8 Structure formula of de-epoxy DON (a) and ketonic compound (b) (Jard et al., 2011)

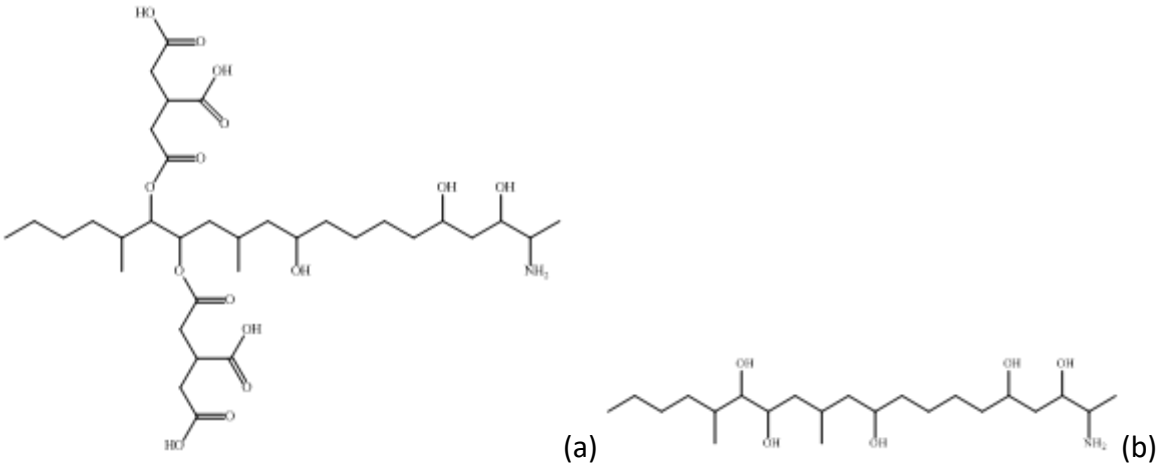

Fig. 2-9 Structure formula of FB_1 (a) and polyolamine (b) (Jard et al., 2011)

2.3.3.2 Adsorption by biological polymer

Microorganism could not only degrade mycotoxins, but also remove them by adsorption to their cell walls. Most gram-positive bacteria and yeasts have demonstrated the adsorption capability. Recent studies are presented in Table. 2-4. It could be observed that microorganism could adsorb 20 to 90% mycotoxins in different liquid food system or even body environment. It has been suggested that heat-treated microorganisms (inactive microorganisms) showed similar or even higher mycotoxin adsorption capability when compared to live microorganisms in aqueous solution (El-Nezami et al., 2002; Turbic et al., 2002; Vosough et al., 2014). It was suggested that the adsorption was through physical adsorption rather than through a biological degradation mechanism. Thus, mycotoxins would not have the chemical reaction with binder during adsorption. This interaction usually occurs with the cell walls of microorganisms. For example, cell walls or peptidoglycans (purified from cell walls) were isolated from lactic acid bacteria (Sreekumar and Hosono, 1998; Zou et al., 2012; Zhao et al., 2013), and were found to bind more toxins than cell pellets after removing the cell walls. The peptidoglycan played an important role in adsorption, and chemical methods can increase the number of adsorption sites and adsorption efficiency (e.g. acid treatment, heat treatment) (Zou et al., 2012). Haskard et al. (2000) suggested that the addition of urea (an anti-hydrophobic agent) or organic solvent destroyed the cell wall toxin complex, proving hydrophobic effect between the adsorptions. For yeast, there were two layers in its cell wall, an inner layer of *β*-1,3-glucan and chitin and an outer layer of *β*-1,6-glucan

with heavily glycosylated mannoproteins (Petruzzi et al., 2014). At the pH range of wine, mannoproteins had negative charges, and OTA carried a positive charge of the amine function (NH_3^+), so that cell wall and toxin could partially establish electrostatic and ionic interactions. Moreover, as a less polar mycotoxin, OTA could bind with hydrophobic surfaces of yeast cell wall through the phenol group and via interactions of two-π-electron orbital (Caridi et al., 2006). However, the adsorption was relatively weak, because toxin-microbe complexes would release toxins about 25 to 40% after washing with PBS buffer (Zou et al., 2012; Fernandez Juri et al., 2015). This might indicate that the adsorption hardly occurs in nonpolar circumstances.

Most microorganisms with adsorption property belong to fermentation microorganisms, thus biological adsorption usually occurs in the process of fermentation in practical production. Food fermentation is a process of decomposing carbohydrates to alcohol or organic acids by microorganisms in aerobic or anaerobic environments, used in the production of fermented dairy products, wine, vinegar, and bread making. The raw food materials that are commonly used for fermentation cover most food groups including dairy, meat, fish, vegetables, fruits, legumes, and cereals (Bourdichon et al., 2012), which could be contaminated by mycotoxigenic fungi or metabolic mycotoxins. Therefore, the mycotoxins are generally present in the fermentation process. The adsorption of mycotoxins by microorganisms during fermentation is summarized in Table. 2-4.

Recently, more attention has been focused on the animal polysaccharides. Chitin, from shrimp shells, was investigated for its ability to bind with AFM_1. Assaf et al. (2018) demonstrated that chitin bound to 17 to 54% AFM_1 in PBS buffer, depending on concentration of both chitin and toxin and incubation time. High adsorption efficiency relied on high chitin concentration and long incubation. A total of 0.15 g/mL of ground shrimp shell or 0.25 g/mL of unground shrimp shell showed more than 90% of the adsorption rates when the incubation was up to 24 h. By contrast, both ground and intact shrimp shell had lower adsorption rates than extracted chitin at same concentration and incubation time. However, the adsorption was not stable binding. After three times washing with buffer, AFM_1 adsorption rate decreased about 15 to 45% in different groups, which suggested the implication of electrostatic bounds (e.g. hydrogen bonds, Van der Waals interactions) in adsorption process.

Due to the presumed environmental and health friendliness of natural products (e.g., enzyme and microorganism cell wall), these approaches have attracted attention. Biological control tends to be costlier than physical and chemical approaches, and there is currently little evidence of the toxicity of enzymolysis products. However, reduction of mycotoxins during production of fermented products would permit the use of somewhat contaminated raw materials in their production.

Table. 2-4 Mycotoxins detoxification by bacteria and fungi through adsorption in different matrixes

Micro-organism	Mycotoxin	Genus	Strains	Effects	Time	Matrix	Reference
Bacteria	AFB_1	*Lactobacillus*	*L. fermentum*	61% Adsorption	48 h	PBS Buffer	Fazeli et al. (2009)
			L. plantarum	56% Adsorption	48 h	PBS Buffer	
			L. casei	48% Adsorption	48 h	PBS Buffer	
			L. paracasei LOCK 0920	Decreased the extent of DNA damage	14 d	*In vivo* (Chicken Fodder)	Slizewska et al. (2010)
				Decreased the extent of DNA damage			
			L. brevis LOCK 0944	Decreased the extent of DNA damage	14 d	*In vivo* (Chicken Fodder)	
				70% Adsorption			
			L. plantarum LOCK 0945 (mixed)		14 d	*In vivo* (Chicken Fodder)	
			L. rhamnosus strain GG		24 h	PBS Buffer	Vosough et al. (2014)
		Bifidobacterium	*B. bifidum*	55% Adsorption	72 h	PBS Buffer	Hamad et al. (2017)
			B. lactis CSCC 5094	35% Adsorption	24 h	PBS Buffer	Peltonen et al. (2001)
			B. longum CSCC 5304	38% Adsorption	24 h	PBS Buffer	
			B. animalis CSCC 1941	46% Adsorption	24 h	PBS Buffer	
			B. lactis CSCC 1906	49% Adsorption	24 h	PBS Buffer	
		Enterococcus	*E. faecium* MF4	23% Adsorption	24 h	PBS Buffer	Fernandez Juri et al. (2014)
			E. faecium GJ40	21% Adsorption	24 h	PBS Buffer	Topcu et al. (2010)
			E. faecium M74	19.3 to 30.5 % Adsorption	48 h	PBS Buffer	
			E. faecium EF031	23.4 to 37.5% Adsorption	48 h	PBS Buffer	
	AFB_2	*Streptococcus*	P. *freudenreichii* sp. *shermanii* JS (mixed)	83% Adsorption	4 weeks	*In vivo* (human)	El-Nezami et al. (2000)
	AFM_1	*Lactobacillus*	*L. rhamnosus* GAF01	95% Adsorption	24 h	PBS Buffer/Milk	Abbes et al. (2013)
			L. plantarum MON03	77% Adsorption	24 h	PBS Buffer /Milk	
			L. plantarum MON03	16% Adsorption	14 d	*In vivo* (mice)	
			L. bulgaricus	58.5% Adsorption	6 h	Yogurt	El Khoury et al. (2011)
			L. bulgaricus	55% Adsorption	6 h	PBS Buffer	
	DON	*Lactobacillus*	*L. plantarum* strain 102	20% Adsorption	24 h	PBS Buffer	Zou et al. (2012)
			L. rhamnosus GGATCC 53103	54% Adsorption	24 h	MRS Medium	Niderkorn et al. (2006)
			*L. delbruekii*s sp. *Bulgaricus*R0149	55% Adsorption	24 h	MRS Medium	
	OTA	*Lactobacillus*	*L. rhamnosus* strain GG	47% Adsorption	2 h	PBS Buffer	Turbic et al. (2002)
			L. rhamnosus strain LC-705	36% Adsorption	2 h	PBS Buffer	
	ZEN	*Lactobacillus*	*L. rhamnosus* GG	55% Adsorption	24 h	MRS Medium	El-Nezami et al. (2002)
			L. rhamnosus LC705	55% Adsorption	24 h	MRS Medium	
	FB	*Lactobacillus*	*L. rhamnosus* GG ATCC 53103	54% Adsorption	24 h	MRS Medium	Niderkorn et al. (2006)

			L. plantarum R1039	40% Adsorption	24 h	MRS Medium	
			L. plantarum R0011	30% Adsorption	24 h	MRS Medium	
			L. brevis R0002	32% Adsorption	24 h	MRS Medium	
			*L. acidophilis*R0052	34% Adsorption	24 h	MRS Medium	
			*L. delbruekii*s sp. *bulgaricus* R0149	55% Adsorption	24 h	MRS Medium	
			*L. casei*s sp. *casei* C3	36% Adsorption	24 h	MRS Medium	
		Streptococcus	*Strep. thermophilus* B5	31% Adsorption	24 h	MRS Medium	Niderkorn et al. (2006)
		Lactococcus	*L. lactis* CS 43	23% Adsorption	24 h	MRS Medium	Niderkorn et al. (2006)
			L. lactis CS 202	40% Adsorption	24 h	MRS Medium	
			L. lactis CS 197	23% Adsorption	24 h	MRS Medium	
		Leuconostoc	*L. mesenteroides* R1107	46% Adsorption	24 h	MRS Medium	Niderkorn et al. (2006)
		Lactobacillus	*L. rhamnosus* 6149	51.1 to 52.0% Adsorption	24 h	Physiological saline solution (0.85%, w/v)	Hatab et al. (2012a)
		Bifidobacterium	*B. bifidum* 6071	52.9 to 54.1% Adsorption	24 h	Physiological saline solution (0.85%, w/v)	Hatab et al. (2012a)
		Enterococcus	*E. faecium* 21605	64.5% Adsorption	24 h	Apple juice	Hatab et al. (2012b)
			E. faecium M74	15.8 to 41.6% Adsorption	48 h	PBS Buffer	Topcu et al. (2010)
			E. faecium EF031	9.5 to 45.3% Adsorption	48 h	PBS Buffer	
Fungi	AFB_1	*Saccharomyces*	*S. cerevisiae*	48% Adsorption	1 h	PBS Buffer	Campagnollo et al. (2015)
	DON	*Saccharomyces*	*S. cerevisiae*	12% Adsorption	1 h	PBS Buffer	Campagnollo et al. (2015)
	PAT	*Saccharomyces*	*S. cerevisiae* strain YS3 (laboratory-prepared)	70% Adsorption	24 h	Apple Juice	Guo et al. (2011)
				76% Adsorption	24 h	Apple Juice	Yue et al. (2011)
			S. cerevisiae strain YS3 (commercial)	50 to 7% Adsorption	24 h	Apple Juice	Guo et al. (2012)
			S. cerevisiae YS1-YS10	100% Adsorption	36 h	Apple Juice	Coelho et al. (2008)
			S. cerevisiae YS3	90 to 96% Adsorption	143 h	Apple Juice	
			S. cerevisiae				
	OTA	*Saccharomyces*	*S. cerevisiae var. boulardii* ATCC MYA-796	39% Adsorption	1 h	PBS Buffer	Petruzzi et al. (2016)
			S. cerevisiae BM45	39% Adsorption	1 h	PBS Buffer	
			S. cerevisiae W13	39% Adsorption	1 h	PBS Buffer	
			S. cerevisiae W28	39% Adsorption	1 h	PBS Buffer	
			S. cerevisiae W47	42% Adsorption	4 d	YPG Medium with Ethanol	Petruzzi et al. (2012)
			S. cerevisiae Y28	37% Adsorption	4 d	YPG Medium with Ethanol	
			S. cerevisiae Malaga LOCK 0173	85% Adsorption	10 d	Grape/Blackcurrant Juice	Piotrowska et al. (2013)
			S. cerevisiae Syrena LOCK 0201	83% Adsorption	10 d	Grape/Blackcurrant Juice	
			S. cerevisiae bakery BS strain	64% Adsorption	10 d	Grape/Blackcurrant Juice	
			S. cerevisiae RC008	57% Adsorption	1 h	PBS Buffer	Armando et al. (2012)
			S. cerevisiae RC009	67% Adsorption	1 h	PBS Buffer	

		S. cerevisiae RC012	71% Adsorption	1 h	PBS Buffer	
		S. cerevisiae RC016	74% Adsorption	1 h	PBS Buffer	
		S. cerevisiae	76% Adsorption	90 d	White Wine	Csutorás et al. (2013)
		S. cerevisiae	86% Adsorption	90 d	Red Wine	
		S. cerevisiae	90% Adsorption	90 d	Rose Wine	
		S. cerevisiae	59% Adsorption	1 h	PBS Buffer	Campagnollo et al. (2015)
		S. cerevisiae	30% Adsorption	1 h	Dough Fermentation	Valle-Algarra et al. (2009)
ZEN	*Saccharomyces*	*S. cerevisiae* RC008	21% Adsorption	1 h	PBS Buffer	Armando et al. (2012)
		S. cerevisiae RC009	33% Adsorption	1 h	PBS Buffer	
		S. cerevisiae RC012	29% Adsorption	1 h	PBS Buffer	
		S. cerevisiae RC016	34% Adsorption	1 h	PBS Buffer	
		S. cerevisiae	75% Adsorption	1 h	PBS Buffer	Campagnollo et al. (2015)

2.3.4 Combined approaches

Pérez-Flores et al. (2011) showed that the level of AFB_1 and AFB_2 in tortillas (a Mexican food) decreased 68 to 84%, according to different original concentration, by microwave treatment (1650 W, 2450 MHz, 5.5 min) with added $Ca(OH)_2$ (0.5%). Kim et al. (2018) reported that among food-grade additives (sodium bicarbonate, vinegar, mixture of sodium bicarbonate and vinegar, citric acid and baking powder), sodium bicarbonate yielded significantly higher PAT reduction in apple juice (from 94.11 to 7.55 μg/L), which was comparatively similar to 30 min of UV irradiation. The authors suggested that because irradiation requires a special UV-irradiation apparatus and energy consumption, a food-grade additive sodium bicarbonate might be a useful alternative to UV radiation for reducing PAT content in apple juice samples. However, sodium bicarbonate treatment affected quality attributes including soluble solids, pH, and colour of apple juice. Nevertheless, the colour and odour of juice treated with sodium bicarbonate could be recovered via addition of citric acid. The usage of some additives could contribute to the mycotoxin destruction in high-temperature processing. Sugars had a positive effect on FB_1 reduction of maize muffins baked at 200℃ for 30 min. In this processing, the influence of glucose (40%) on the decrease of FB_1 was greater than that of fructose (27%) and sucrose (28%), and the effect of glucose concentration was more significant, from 40% reduction for 0.075 g glucose/g maize meal to 52% reduction for 0.3 g glucose/g maize meal (Castelo et al., 2001). Also in this study, Castelo et al. (2001) showed that when grits with added sugars of different concentration (2.5% and 5%) were extruded at a screw speed of 80, 100 or 120 rpm, the amounts of FB_1 remaining were around 40 to 80% at 140℃. A total of 10% glucose with 40 rpm of extrusion at 160℃ led to about 90% reduction of FB_1, which was about 20% higher than the FB_1 treated without glucose (Voss et al., 2011). In another extrusion study with conditions of sample moisture (15% or 30%), screw speed (120 rpm), temperature (150℃ or 180℃) with or without 1% sodium metabisulphite addition, DON was significantly inactive (>95%) in maize flour treated under every condition, but AFB_1 content was not greatly affected (10 to 25%). Compared to glucose, sodium metabisulphite did not show a significant contribution to the reduction of both DON and AFB_1 (Cazzaniga et al., 2001). With the addition of 30 mL of lemon juice and 6 g of citric acid, AFB_1 deceased up to 93.1% in 50 g pistachio nuts. When lemon juice and citric acid reduced to 15 mL and 2.25 g, respectively,

only 49.3% of AFB_1 could be detected (Rastegar et al., 2017). Furthermore, adding baking soda under twin-screw extrusion could contribute to the reduction of OTA in oat-based food, and the degree of content reduction improved form about 40 to 65% with the increase of added soda from 0 to 1%. On the contrary, the baking soda did reduced OTA by a modest 10% in rice-based food (Ryu et al., 2019). The degradation of PAT with added ascorbic acid was predicted by nonlinear Weibull model to be higher than that without ascorbic acid, and the degradation increased with the raising of temperature. This might be because free radicals formed from oxidized ascorbic acid attacked the lactone structure of PAT (Kokkinidou et al., 2014).

The exploration of combined treatments is to pursue higher removal efficiency, taking advantage of the additional effect of integrated management. This is becoming a trend gradually. The combined treatments do reduce toxin contamination to a greater extent, and they can better adapt to different food matrixes.

2.4 Evaluation of the feasibility of the approaches to be applied to food production

Several reports have shown the potential of various methods for preventing and reducing fungi or mycotoxins in foods. Here, we perform a comparative evaluation of all the methods discussed in this review, on the basis of their technical advantages and disadvantages, the food matrixes for which each method is suitable, the safety concern of a method, and the economical implication of large-scale application. Using all these parameters, the potential for upscaling each method is then estimated as high, medium, or low (Table.2-5).

In general, physical approaches, which include temperature and humidity control, MA treatment, irradiation treatment, cleaning, milling and sorting, and heat treatment, showed medium to high potential for using at a large scale. The advantages include versatility to use in various matrixes, safety, and few changes to the nutritional and sensory properties of foods. However, the high cost of equipment and high energy required to operate over long times may limit the industrial

deployment of these methods. Among the four methods, temperature and humidity control appears to have the highest upscaling potential.

Chemical approaches include photodynamic treatment, plasma treatment or ozonization, EOW, chemical antifungal/anti-mycotoxins agents, and chemical removal of mycotoxin. Application of these methods in large scale also showed a medium to high potential. This can be justified by their high efficiency and their suitability for a wide range of food matrices. The limitations are mainly due to the negative impact on the quality and safety of foods. The use of EOW showed the highest potential for large-scale application with low level of safety concern, once the cost of EOW production equipment and energy can be lowered.

Biological approaches include methods such as the use of BCAs, use of antifungal plant metabolites, and biological removal of mycotoxins. These approaches showed low to medium potential for upscaling. They are claimed to be environmental friendly, they have a high efficiency (although the replication of lab performance of BCAs in the field remains a challenge), and they can be applied to various foods of plant and animal origin. However, these methods may actually food quality; the binders are difficult to remove from food and feed, the potential toxicity can be high, and the cost of production of the biological or plant agent is also high. Among the three methods, the biological removal of mycotoxins, which is already largely used in feed industry, shows a great prospect.

Table. 2-5 Evaluation of fungi/mycotoxin decontamination approaches

Classification	Treatments	Technical advantages	Technical disadvantages	Suitable food matrix	Safety	Economical concerns in large scale production	Potential for using in large scale	Reference
Physical approaches	Temperature and humidity control	Easy-operated; less colour, odour and nutrition changes; shelf-life extension	Inconvenient transportation	Almost all food types	No reported toxic substance induced and formed	Cost of temperature and humidity equipment; high energy consumption	High	-
	Modified atmosphere treatment	Less colour, odour and nutrition changes; shelf-life extension	Large consumption of packaging material	Packed foods	No reported toxic substance induced and formed	Cost of gas generator equipment and food packaging material	Medium	-
	Irradiation treatment	High efficiency; environmental friendly; agrees with the legislations of food application in 55 countries	Food quality change (e.g. colour, odour); nutrition loss (e.g. oxidization of vitamin) at high dose	Packed foods; frozen foods; liquid foods; cereals; fruits and vegetables	No residual irradiation; may cause mutations to fungi	Low consumption of water and electrical energy (exception of electron beam and X-ray); high cost of food irradiation facilities	High	Calado et al. (2014)
	Cleaning, milling and sorting	Easy-operated; effective with water-soluble mycotoxins	Less effective with organic-soluble mycotoxins	Raw food materials	No other introduced chemicals and new mycotoxin-derivative	High consumption of water	High	Temba et al. (2016b)
	Heat treatment	Already a necessary processing method in food production	Change of the desired physical properties of food	Cooked foods (e.g. roasted foods); sterilized food	Lack of studies on transformation mechanisms	Cost of heating equipment; high energy consumption	Medium	Rastegar et al. (2017)
Chemical approaches	Photodynamic treatment	Environmental friendly; biochemically stable; photosensitizer adequately activated by using easy-available visible light	Limited light penetration	Cereals; fruits; sea foods; animal feeds	Food grade photosensitizer (e.g. curcumin); lack of studies on safety after treatment	Cost of light generator and photosensitizer; relative cost-effective		Njoki et al. (2017) Al-Asmari et al. (2018) Temba et al. (2019)
	Plasma treatment (Ozonisation)	High efficiency; rapid; no significant change of nutritional components to whole cereals	May cause the loss of nutrition in other foods; change of colour; production of undesirable odour	Cereals; meat; fruits and vegetables; herbs and spices; animal feeds	Lack of studies on safety of degraded residue	Low energy consumption; high cost of cold plasma production equipment; less maintenance and dust cleaning	Medium	Savi et al. (2015a) Temba et al. (2016b) Misra et al. (2019) Alexandre et al. (2017)
	Electrolyzed oxidizing water	High efficiency; environmental friendly; easy-operated	Loss of antifungal activity without continuous electrolysis; Cl_2 production; possibility of metal corrosion	Fruits and vegetables; meat products; cereals	Safe to degrade mycotoxins; no not corrosive to skin, mucous membrane and organic material	High cost of EOW production equipment, electrical and water	High	Okull and Laborde (2004) Zhang et al. (2012) Huang et al. (2008)

						consumption; low cost of each litre		
	Chemical antifungal/anti-mycotoxins agents	Effective; easy-operated	Unpleasant chemical residue	Coating; animal feeds; specific food conforming to food additives (e.g. soda in Chinese baking)	Lack of studies on transformation mechanisms; toxicity of induced chemicals at high concentration to human and environment	Cost of agents	Medium	Bretz et al. (2006) Temba et al. (2019)
	Chemical removal of mycotoxin	Effective; easy-operated; some commercial clay materials enhance nutrition and digestibility of animal feeds	Difficult removal of mycotoxin-binder complex; need to be in aqueous environment	Animal feeds; clay capsules for human (potential)	Toxicity of released mycotoxins from mycotoxin-binder complex	Cost effective	Medium	Di Gregorio et al. (2014)
Biological approaches	Biological control agents/mycotoxin degradation	High efficiency in lab experiments; easy-operated (e.g. soak, spray); environmental friendly	Less evidence on the correlation between laboratory inhibition assay and field performance; need to be under strict conditions (e.g. pH, solution, temperature)	Cereals; fruits and vegetables; fermented foods	Less toxicity shown on degraded residue	Cost of bacterial high-density culture; used as antagonist solution cost of materials and equipment for production of the biocontrol agent	Low	Jard et al. (2011) de Medeiros et al. (2012)
	Antifungal plant metabolite	High efficiency; a wide range of sources	Change of colour and odour; mainly used in aqueous environment	Meat products; dairy products; vegetable and fruits; cereals	Potential toxicity (e.g. carcinogenicity) at high concentration	Large amount of plant materials needed; high cost of production equipment and energy consumption	Low	Burt (2004) Bakkali et al. (2008)
	Biological removal of mycotoxin	High efficiency; from food source; environmental friendly	Difficult removal of mycotoxin-binder complex; need to be in aqueous environment	Fermented foods; animal feeds	Toxicity of released mycotoxins from mycotoxin-bacteria complex	Cost of bacterial high-density culture	Medium	Hathout and Aly (2014)

2.5 Conclusion and perspectives

In order to reduce the contamination of foods by mycotoxins and minimize their negative effects on consumers health, 15 of strategies have been reviewed, which can be classified into physical, chemical, and biological approaches, singly or in combination. These strategies are mainly focused on control of fungi growth in raw food materials and removal of mycotoxins from foods. Some new and efficient methods, such as plasma and EOW treatment, show great potential but currently remain limited to laboratory applications. Currently, physical approaches can be adapted into a wider range of food matrixes, including dry or liquid, raw or cooked foods. Physical approaches can be applied at large scale (e.g., crop storage) and small scale (MAP). Chemical and biological approaches are usually applied in high humidity conditions (e.g., coating of fruits and vegetables) or liquid environment (e.g., mycotoxin binders in wine). However, so far no single approach is universal for all matrices or 100% effective at removing the risk of aflatoxin contamination. With the increasing demands in food safety and advances in technology, the mycotoxin reduction strategy has become to multidimensional, including a combination of multiple control methods as an integrated management strategy. Food safety concerns as a result of these treatments remain. It is critical when developing or applying a method to test the toxicity of the applied agents and the derived secondary products. Nutrient loss and deterioration of sensory properties of foods by methods such as irradiation and plasma treatment must be tackled. For biological control methods, the efficacy of the BCAs in field condition must be proven, and their short- and long-term toxicity be monitored. In methods involving plant-extracted metabolites, large amount of plant materials is needed to obtain sufficient amount of metabolites. Valorization of plant waste can offer an alternative low-cost source of the plant metabolites. On the other hand, the high cost of equipment and running limits the industrial application of most of the methods, whereas low cost and easy operations such as sorting and cleaning can be upscaled if they are mechanized. This underlines the need of multidisciplinary collaboration involving engineering, physical, and biological sciences in the fight against mycotoxins. Moreover, environmental aspects must be considered during the disposal of toxin-contaminated sorted seeds, waste water, or binders.

Research usually remains at the laboratory level with little consideration for upscale applications. Physical approaches have shown the highest potential for upscaling, followed by chemical approaches, whereas biological approaches necessitate further improvements. Finally, in addition to developing mycotoxin reduction methods, educating producers and consumers on the toxicity of mycotoxins, improving the diversity of food choices (to prevent acute doses from single sources such as maize or rice), and guiding to change the food preferences (toward foods that are less prone to mycotoxin contamination) can also reduce the harmful effect of mycotoxins from human health.

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Chapter 3: Green extraction of polyphenols from citrus peel by-products and their antifungal activity against *Aspergillus Flavus*

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 to 400 $mg \cdot mL^{-1}$, 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-DAD, narirutin and hesperidin were the most abundant. In conclusion, citrus peels are promising bioresources of antifungal agents with potential applications in food and other industries.

Key words: Antifungal activity; *Citrus* sp.; *Aspergillus flavus*; Polyphenols; Solid Phase Extraction

3.1 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 et al., 2019). A good approach to prevent aflatoxins in food is

by inactivating *A*. *flavus* and preventing its growth (Rasheed et al., 2020). The usage of biological metabolites to prevent fungal growth is regarded as a safe, effective and environmental-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, more than 140 million tons of citrus were produced in 2019 (FAOSTATS, 2021). Apart from fresh produce, citrus fruits are processed into juice, canned or dehydrated products, marmalades, jams, and flavouring agents. Around 50 to 60% of the fruit weight, including peels, seeds and segment membranes, are generated as by-products after processing (Mahato et al., 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 et al., 2004). Flavonoids are the most common group of polyphenols in plants, playing important roles in plant responses (Xi et al., 2014) and also showing antifungal activities (Al Aboody and Mickymaray, 2020). The most abundant flavonoids in citrus have been identified as naringin, hesperidin, narirutin, and neohesperidin (Xu et al., 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 et al., 2018), and food spoilage (Ben Hsouna et al., 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 $mg \cdot mL^{-1}$, hesperidin at 153 $mg \cdot mL^{-1}$ and neohesperidin at 153 $mg \cdot mL^{-1}$) could inhibit growth of *A. flavus* by 33 to 41% (Salas et al., 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

bioactive. Most studies that investigated extraction of polyphenols from plant materials used organic solvents (Karim et al., 2016; Olakunle et al., 2019; Mohotti et al., 2020). 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.

3.2 Material and methods

3.2.1 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°C and then micronized to particle size of < 150 µm. All the samples were packed in polythene bags and stored at -20°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, quercitrin, rutin, isorhoifolin, rhoifolin, luteolin, cymaroside, didymin, poncirin, eriodictyol, eriocitrin, neoeriocitrin, 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 SΛFC (USA), vanillic acid from Alfa Aesar (USA), and caffeic acid from Cayman Chemical (USA).

3.2.2 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 $g \cdot L^{-1}$ potato dextrose broth (PDB, Sigma, UK), for 2 days. Culture medium (0.5 mL) containing conidia was spread on 15 mL of 39 $g \cdot L^{-1}$ potato dextrose agar (PDA, Sigma, UK) in 9 mm petri dish, the dish was incubated at 30°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.

3.2.3 Citrus peel extraction

Ten grams of dried citrus peel powder were extracted in 200 mL of absolute ethanol or water at 60°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°C.

3.2.4 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). In order to improve the separation efficiency, second SPE was performed for 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 (Heidolph, Germany) at 60°C, and then freeze-dried. All samples were stored at -20 °C until use.

3.2.5 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 1mL of 10% DMSO and added to 15 mL molten PDA at around 50°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 of *tert*-Butylhydroquinone (TBHQ) as positive control. To avoid microbial contamination, the PDA medium with extracts was autoclaved at 121°C for 15 min before pouring into petri dishes. Autoclaving was found not to affect antifungal activity significantly (Fig. A-1, Table. A-12) 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°C for 7 days. The diameter of the fungal colony was measured every day.

The antifungal activity was calculated as: $\text{Antifungal activity } (\%) = \left(\frac{Dc-Dt}{Dc}\right) \times 100$

Where, *Dt*: the diameter of fungal colony in the treated dishes; *Dc*: the diameter of fungal colony in the negative control dishes.

3.2.6 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.

3.2.7 Identification and quantification of polyphenols

3.2.7.1 Determination of total phenolic content (TPC)

TPC of extracts was determined by the Folin-Ciocalteu reagent method, based on Singleton et al. (1999) with some modifications. Briefly, 10 µL of sample was mixed with 40 µL of Folin-Ciocalteu (12.5%) and 150 µL 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 µL 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).

3.2.7.2 Determination of total flavonoids content (TFC)

TFC of each extract was determined by aluminium chloride colorimetric assay following the method described by Sembiring et al. (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 µL of 50% ethanol solution was used as negative control. Serially 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).

3.2.7.3 HPLC analysis of phenolics compounds

The phenolic compounds were determined by HPLC method as previous 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 x 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. 3-2S).

3.2.8 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.

3.3 Results and discussion

3.3.1 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 $mg \cdot mL^{-1}$ on *A. flavus* mycelia growth is shown in Fig. 3-1. 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. 3-1a). Among the extracts (Fig. 3-1b), mandarin peel extract displayed the highest inhibition activity (ranging from 53 to 40% over 7 days), followed by the orange extract (40 to 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 et al. (2007) who compared the inhibitory effect of five citrus peel extracts, including *C. reticulata*, *C. aurantifolia*, *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 to 71.10%). The higher activities observed by these authors suggests 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.

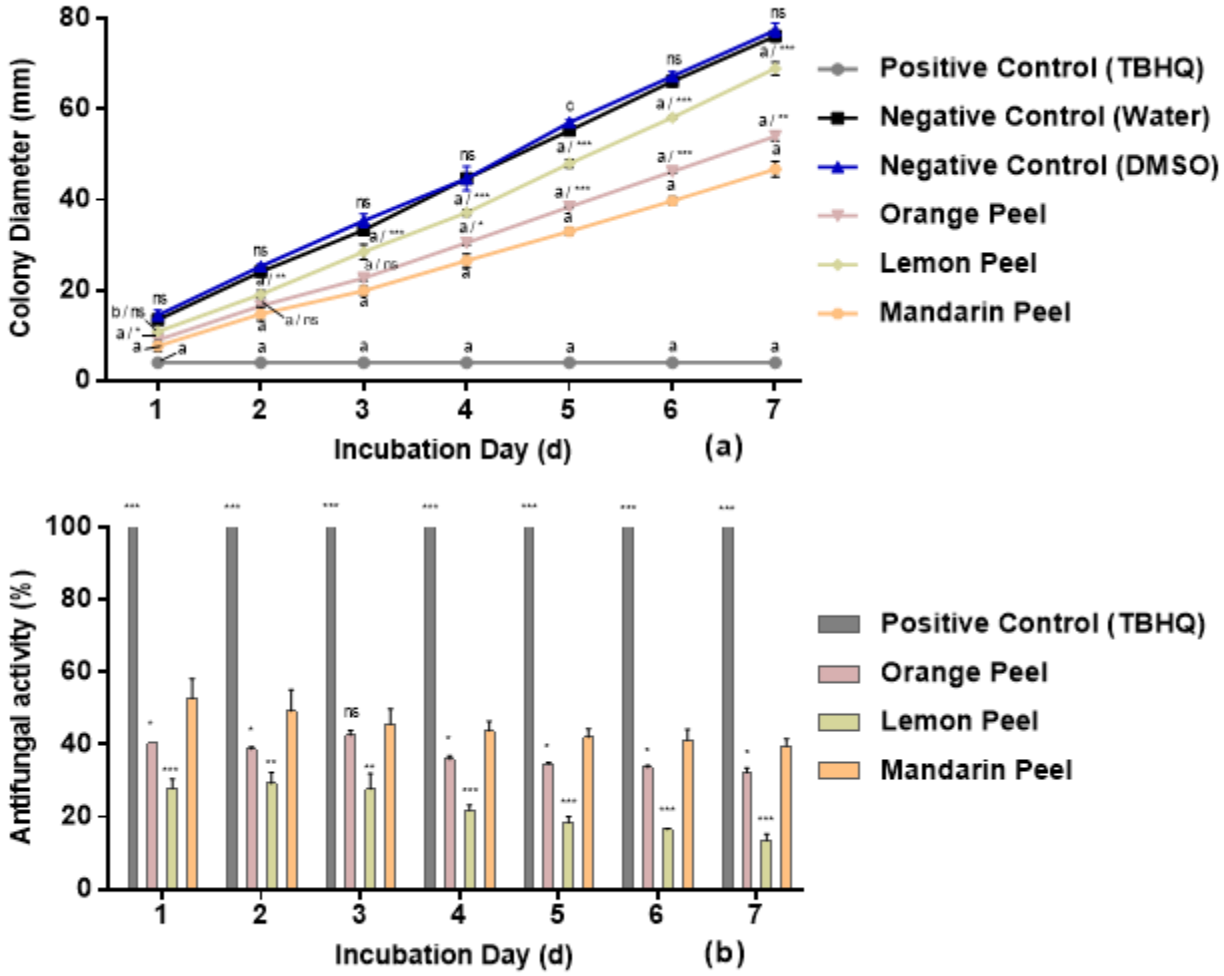


Fig. 3-1 Antifungal activity (%) of ethanol extracts (10 mg/mL) from orange, lemon and mandarin citrus peels, assayed at 30°C for 7 days. Colony diameter (mm) including disc diameter of 4 mm (a) and antifungal activity (%) compared to TBQH positive control (b). 'a, b, c' represent significant differences comparing each peel extracted samples to negative control (DMSO) for colony diameter; * represents significant differences comparing orange and lemon extracts to mandarin extracts. a/***, p≤0.0001; b/**, p≤0.001; c/*, p≤0.05 and ns, p>0.05. Results are means of three replicated dishes.

3.3.2 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 $mg \cdot mL^{-1}$. The results show that the fungal inhibition increased with the

concentration of extracts (Fig. 3-2). *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 (*A. flavus* NNRL 3375, Cranfield University) suggested that both strains performed similarly at 10 mg·mL^{-1} of mandarin extracts (data not shown). Compared to a report by Oikeh et al. (2016) which investigated the effect of citrus juice concentrates, the MIC in the present study was higher. Three citrus juice concentrates were found to completely suppress *A. niger* and *Penicillum* sp. at concentrations ranging 50 to 100 µg/mL. The differences may be attributed to the concentration and pH differences between juice and peel. Some studies also investigated the minimum fungicidal concentration (MFC) which is the lowest concentration required to prevent the formation of any colony forming units (CFUs), usually by killing 99.98% of the inoculum (Prakash et al., 2012; Prakash et al., 2011). The MFC is normally higher than the MIC. MFCs were no considered in this study, as the MICs were found to be already quite high.

It is also worth noting that the inhibitory effect of ethanol extract was higher than that of water extract at each concentration (Fig. 3-2). For instance, at the concentration of 10 mg/mL, ethanol extract inhibited the growth of *A. flavus* by 47% in 7 days, while the inhibition by water extract was about 28%, significantly lower (p<0.05). This result is in agreement with the findings of Ndonkeu Mangoumou et al. (2013) showing that the activity of ethanol extracts from Cameroonian plants was up to 53% higher than cold water extracts at same concentration. However, water extracts still possessed good levels of antifungal activity and water extraction may be a viable, greener option for industry to produce antifungal agents from citrus peel at scale.

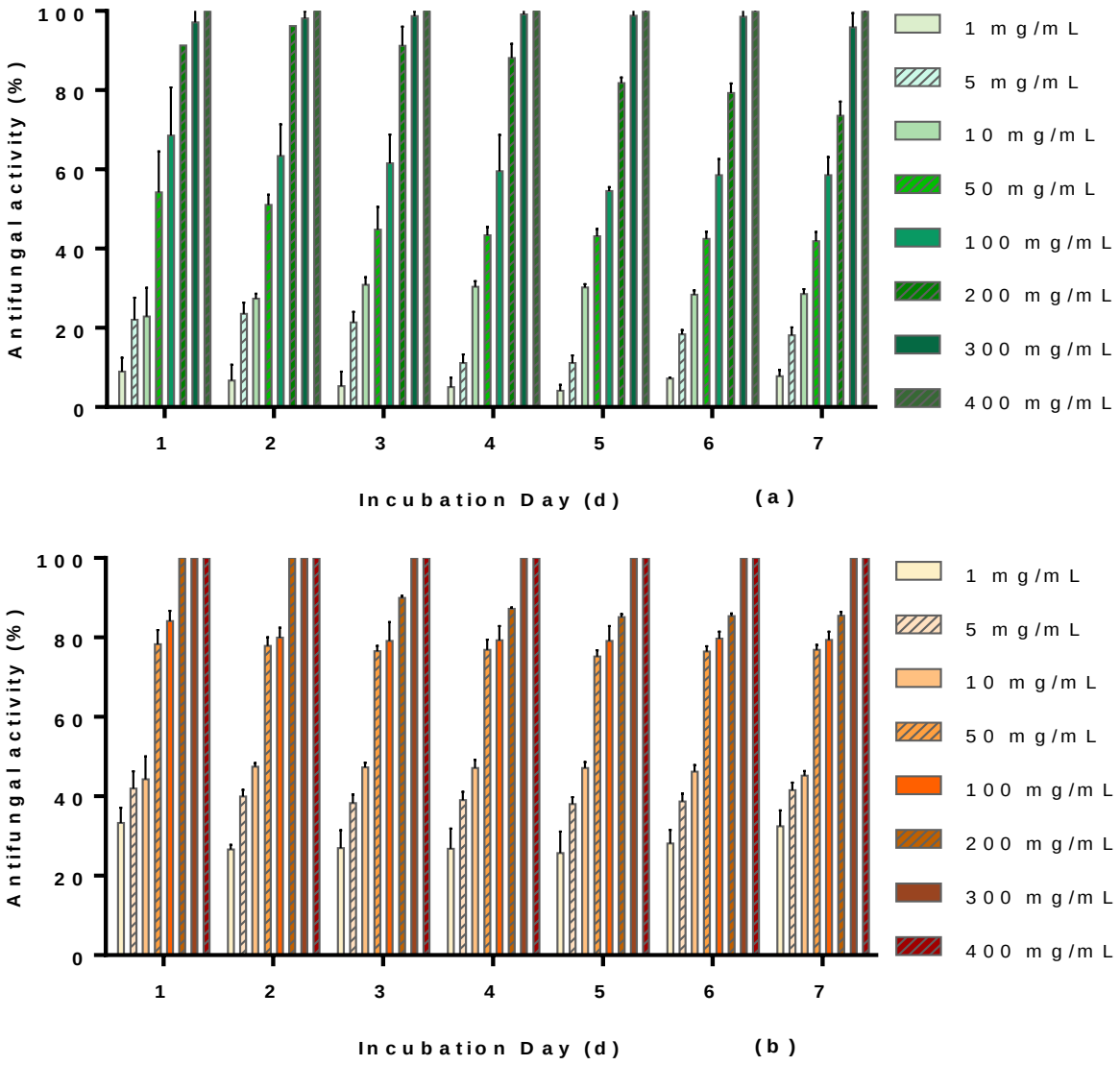


Fig. 3-2 Effect of concentrations (mg/mL) of water (a) and ethanol (b) extract on *A. flavus* colony growth at 30°C during 7 days.

3.3.3 Antifungal activity of mandarin extracts prepared by SPE

As shown in Fig. 3-3, the elution fraction (EF) of water extract (10 mg/mL) inhibited the fungus by 60%; whereas the washing fraction (WF, 10 mg/mL) promoted fungal growth by 4.61 to 19.74% after 7 days. Similarly, the EF of ethanol extract inhibited A. *flavus* growth by 65.13 to 70.47%,

and WF increased fungal growth up to 7.15%. Therefore, SPE technique significantly ($p \leq 0.0001$) 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).

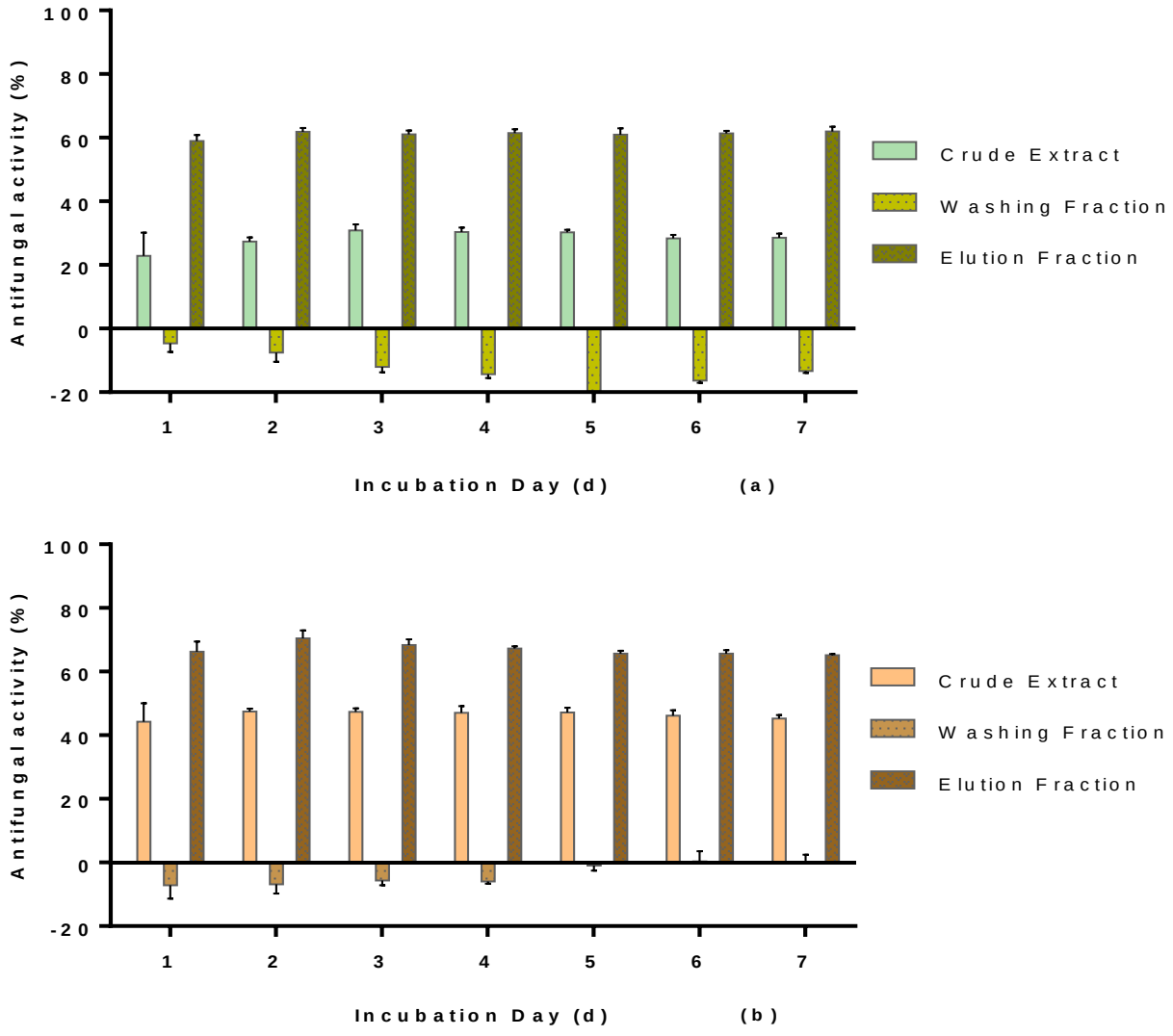


Fig. 3-3 Antifungal activity (%) of water (a) and ethanol (b) mandarin peel extracts and their SPE fractions (10 mg/mL) at 30°C in 7 days.

3.3.4 Identification and quantification of polyphenols in mandarin peel extracts

3.3.4.1 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. 3-1). The results show that water could extract more phenolic compounds than absolute ethanol at 60°C. In contrast, in the study by Lapornik et al. (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 amberlite (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. 3-1. The TFC of water and ethanol in EF were 2470.22 or 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 et al., 2018). On the other hand, methoxylated polyphenols do not react with the reagent (Margraf et al., 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 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 B-ring. If the hydroxyl groups are unavailable due to glycosylation or methoxylation, this would

Table. 3-1 The total phenolic and total flavonoid content in mandarin peel crude and SPE extracts

		Solvent	Crude Extracts		Washing Fractions		Elution Fractions	
	Yield (%)	Water	42.24		33.16		5.68	
		Ethanol	31.88		24.36		4.72	
				Recovery (%)[b]		Recovery (%)		Recovery (%)
Phenolic Content	Content in 1g Peel (μg GAE[a])	Water	6242.80 ± 198.56	100	2822.00 ± 103.52	45.20	3253.87 ± 153.60	52.12
	Content in 1g Peel (μg GAE)	Ethanol	4244.80 ± 222.33**	100	1752.53 ± 24.49	41.29	2457.33 ± 127.27	57.89
Flavonoid Content	Content in 1g Peel (μg RE[a])	Water	3149.78 ± 1.54	100	243.17 ± 39.98	7.72	2470.22 ± 36.59	78.43
	Content in 1g Peel (μg RE)	Ethanol	2080.72 ± 35.18***	100	183.04 ± 30.16	8.80	1749.42 ± 21.34	84.08

[a)] GAE, gallic acid equivalent; RE, rutin equivalent

[b)] Recovery (%), the percentage of each fraction recovered from crude extracts

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.

prevent chelation with $AlCl_3$ and consequently reduce the bathochromic effect. Thus, while flavones and flavonols are able to be detected with $AlCl_3$ method, polymethoxylated flavones (PMFs) can be hardly measured and flavanones give low response to $AlCl_3$ reagent, which might cause underestimation of the amount of TFC in samples (Huang et al., 2018). Therefore, to accurately determine the content of each phenolic compound in the extracts, HPLC analysis of the various extracts was performed.

3.3.4.2 Polyphenol composition analysis of crude and SPE extracts

The polyphenol composition of extracts was analysed by HPLC with characteristic chromatograms for mixed standards (Fig. A-3) and mandarin peel extracts (Fig. 3-4). The compounds identified in each extract are listed in Table. 3-2. They were identified based on their retention time based on major citrus phenolic contents mentioned in the literature (Table. A-11). The chemical structure of these compounds is shown in Fig. 3-5. Mandarin peel extracts contained 12 out of 34 phenolic compounds analysed, including 5 flavanones, 5 PMFs and 2 phenolic acids (Fig. 3-5). These were identified and determined quantitatively.

Water and ethanol crude extracts were predominantly composed of hesperidin (1643.60 μg/g and 1304.77 μg/g) and narirutin (2155.57 μg/g and 2044.46 μg/g). This was partly in agreement with Zhang et al. (2014) and Nogata et al. (2006) which showed high hesperidin content in mandarin fruits, while in this study, narirutin was the most abundant flavonoid in peel extracts. Both crude extracts also contained a non-negligible quantity of rutin (221.90 μg/g and 211.54 μg/g for water and ethanol extracts, respectively), taxifolin (139.17 μg/g and 134.36 μg/g), sinensetin (82.58 μg/g and 113.82 μg/g), nobiletin (121.09 μg/g and 218.02 μg/g), and low amount of eriocitrin (87.24 μg/g and 82.60 μg/g), didymin (52.51 μg/g and 51.95 μg/g), eriodictyol (31.24 μg/g and 31.10 μg/g), tangeretin (40.06 μg/g and 74.83 μg/g). Flavanones are the typical polyphenols usually accounting for a great proportion in citrus polyphenols. In addition to hesperidin, narirutin, eriocitrin and didymin, there are other flavanones including naringin, neohesperdin, poncirin, naringenin identified in different *citrus* sp. (Table. A-13), but were not found in our mandarin extract. Moreover, PMFs rutin, sinensetin, nobiletin and tangeretin, detected in the present study, were also found in other studies. Other PMFs including

isorhoifolin, diosmin, neodiosmin, rhoifolin, luteolin, diosmetin (Table. A-13) were previously found in other species, but not in our mandarin extract.

For phenolic acids, only 2 hydroxycinnamic acids were identified as ferulic acid (67.05 μg/g and 65.47 μg/g) and *p*-coumaric acid (34.00 μg/g and 40.77 μg/g) in both water and ethanol crude extracts. In most of the studies on mandarin phenolic acids, the hydroxycinnamic acid contents were much higher than benzoic acids, which is in agreement with the present study (Xi et al., 2014). Furthermore, there were other hydroxycinnamic acids, benzoic acids and *p*-hydroxybenzoic acid that have been previously identified in various citrus peels (Table. A-13) that have not been found in the mandarin extracts.

These differences in the composition of citrus polyphenols may be caused by the genetic background and/or tissues of fruits, environment factors, and extraction solutions and methods (Nogata et al., 2006; Salas et al., 2011). Therefore, in order to improve the utilization of citrus peel, the extraction technology should be optimized depending on different citrus materials and the target compounds.

There was no big difference in the composition of phenolic compounds extracted by water or ethanol as quantified using HPLC (Table. 3-2). Phenolic compounds are described as amphiphilic molecules because they usually contain hydroxyl groups that can contribute to the hydrophilic nature of the molecules, as well as ring structures that can lead to 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 $Log_{10}P$ values. Hence, amongst the 12 identified polyphenols, *p*-coumaric acid (1.43), sinensetin (3.19), nobiletin (3.37) and tangeretin (3.78) have a relatively higher theoretical $Log_{10}P$ value, which correlated with significantly higher ($p<0.05$) solubility in ethanol than water. On the contrary, the $Log_{10}P$ 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 μg/g respectively) than ethanol extract (1346.44 and 82.60 μg/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 $Log_{10}P$ 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 (Karakaya, 2011; Farooque et al., 2018). Water was used in washing steps to remove polar molecules (e.g. sugars), while ethanol disrupts the interaction between hydrophobic components and the resin that elutes the adsorbate. According to the theoretical $Log_{10}P$ 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 to 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 $AlCl_3$ method, HPLC analysis more completely and accurately reflected the phenolic compounds in citrus extracts. However, the Folin-Ciocalteu and $AlCl_3$ 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.

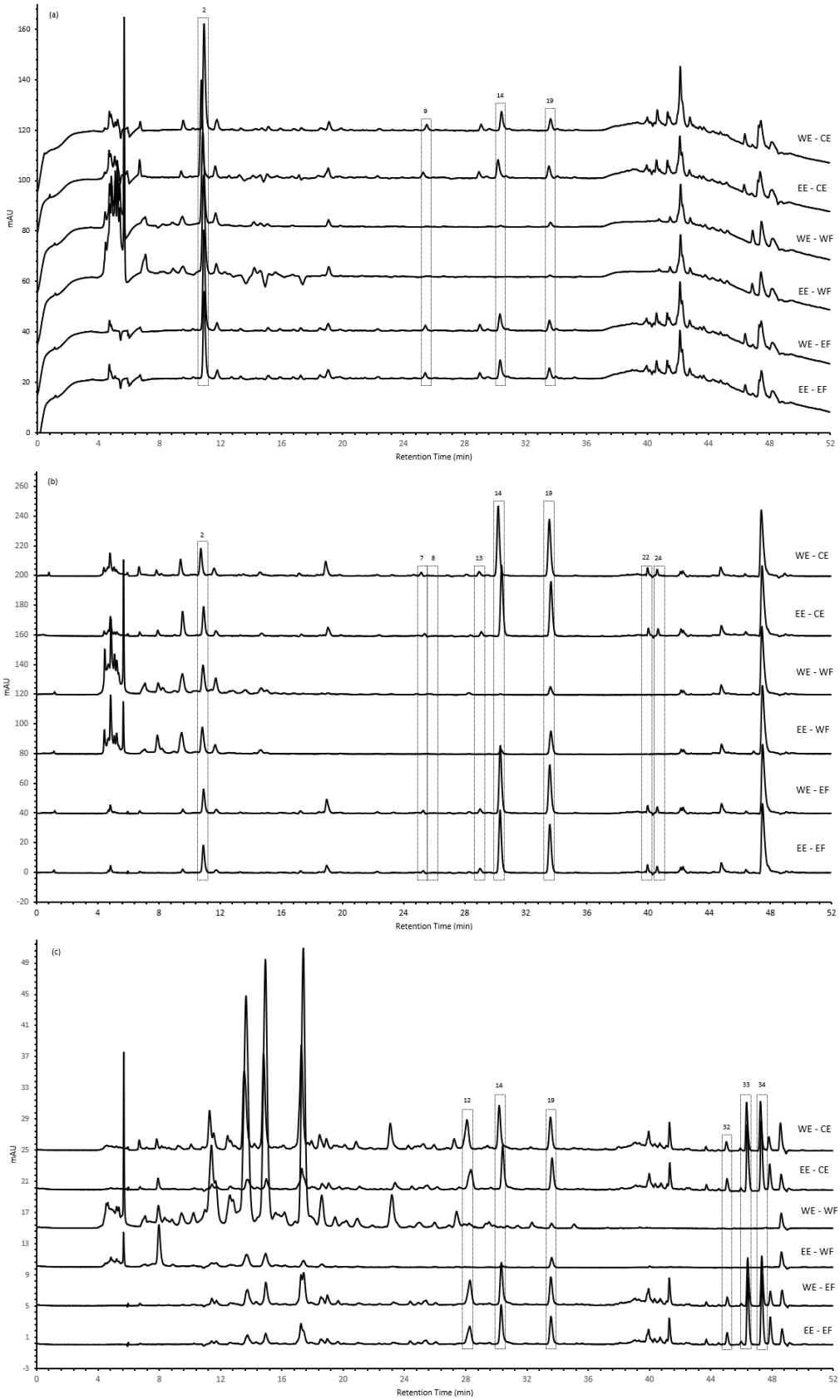


Fig. 3-4 HPLC chromatogram of mandarin peel extracts at 254 nm (a), 280 nm (b) and 330 nm (c). WE, water extract; EE, ethanol extracts; CE, crude extracts; WF, washing fraction; EF, elution fraction. Peaks: 2, Protocatechuic acid (internal standard); 7, ρ-coumaric acid; 8, Eriocitrin; 9, Rutin; 12, Ferulic acid; 13, Taxifolin; 14, Narirutin; 19, Hesperidin; 22, Didymin; 24, Eriodictyol; 32, Sinensetin; 33, Nobiletin; 34, Tangeretin.

Table. 3-2 Phenolic composition (μg/g of mandarin peel) of mandarin peel crude and SPE extracts with their theoretical partition coefficients ($Log_{10}P$)

Peak nº	Compounds	$Log_{10}P$ [b]	Water Extract				Ethanol Extract			
			Crude Extracts	Washing Fractions	Elution Fractions	Recovery (%)[a]	Crude Extracts	Washing Fractions	Elution Fractions	Recovery (%)
7	*p*-coumaric acid	1.43	34.00±0.01***	-	40.30±11.03 ns	118.53	40.77±0.09	-	36.26±0.00	88.94
8	Eriocitrin	-0.86	87.24±0.45***	-	82.76±2.01**	107.15	82.60±0.10	-	64.78±3.34	78.43
9	Rutin	-1.06	221.90±1.28ns	-	211.54±8.38*	95.33	214.50±9.28	-	171.06±4.46	79.75
12	Ferulic acid	1.25	67.05±2.23 ns	-	55.02±4.78 ns	82.06	65.47±4.56	-	60.34±5.57	92.16
13	Taxifolin	0.71	139.17±6.67 ns	-	115.66±1.17 ns	83.11	134.36±3.71	-	108.93±6.03	81.07
14	Narirutin	-0.37	2155.57±74.03 ns	6.76±0.71***	1962.55±30.90 ns	91.05	2044.46±55.48	22.45±1.28	1794.81±68.02	87.79
19	Hesperidin	-0.55	1643.60±66.11*	38.27±1.18***	1304.77±9.46 ns	79.38	1346.44±67.78	100.12±2.29	1231.19±32.72	91.44
22	Didymin	0.17	52.51±4.40 ns	-	52.63±2.15ns	100.23	51.95±44.42	-	57.51±4.53	110.70
24	Eriodictyol	1.63	31.24±0.79 ns	-	30.25±1.44 ns	96.83	31.10±4.11	-	26.72±3.16	85.92
32	Sinensetin	3.19	82.58±3.38**	-	76.90±1.18***	93.12	113.82±4.73	-	105.30±1.95	92.51
33	Nobiletin	3.37	121.09±0.07***	-	121.07±0.11***	99.98	218.02±7.29	-	203.81±4.76	93.48
34	Tangeretin	3.78	40.06±1.93***	-	37.66±0.53***	94.01	74.83±3.01	-	70.90±2.12	94.75
	Total		4676.02	45.03	4091.11		4418.32	122.57	3931.62	

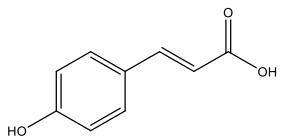
[a] Recovery (%), the percentage of EF recovered from crude extracts

[b] Theoretical partition coefficients ($Log_{10}P$), Theoretical log10P theoretical values were obtained via online logP calculator 'molinspiration', on Web site: https://molinspiration.com/services/logp.html (accessed in May 2021). The Canonical SMILES used in logP calculation was obtain from 'PubChem', on Web site: https://pubchem.ncbi.nlm.nih.gov/ (accessed in May 2021).

(-) represents the component was not detected in extracts

Data are presented as means ± SD of triplicate samples. * represents significant differences comparing ethanol extracts to water extracts. ***, p≤0.0001; **, p≤0.001; *, p≤0.05; ns, p>0.05.

(a)



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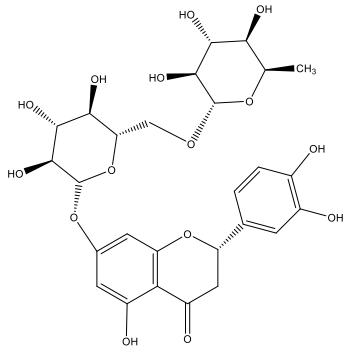

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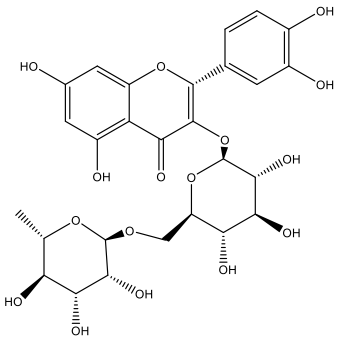

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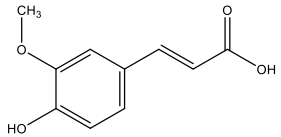

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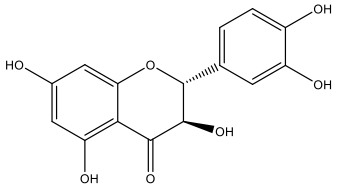

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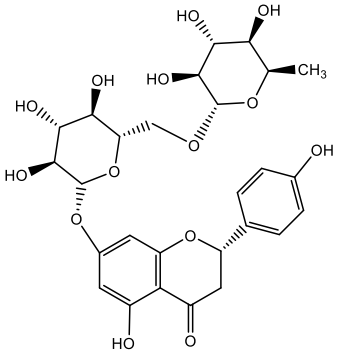

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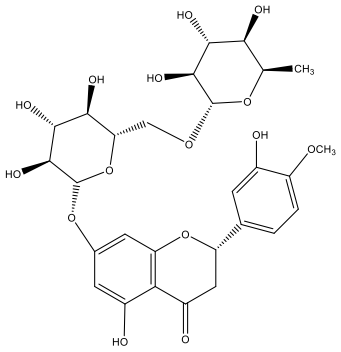

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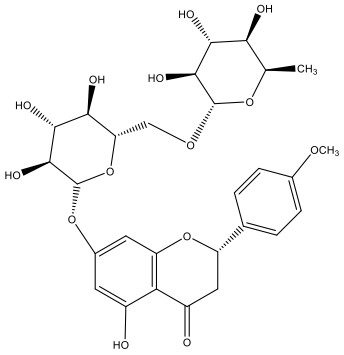
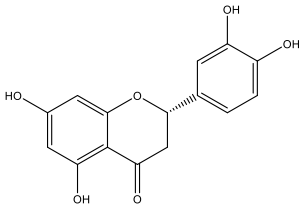
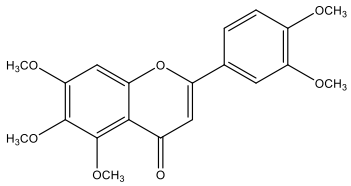
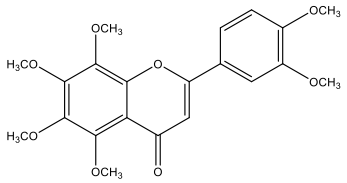


(i)



(j) (k) (l)





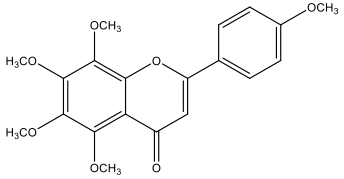


Fig. 3-5 Chemical structures of p-Coumaric acid (a), Eriocitrin (b), Rutin (c), Ferulic acid (d), Taxifolin (e), Narirutin (f), Hesperidin (g), Didymin (h), Eriodictyol (i), Sinensetin (j), Nobiletin (k) and Tangeretin (l).

3.3.5 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) (Sanver et al., 2016; Makarewicz et al., 2021). 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 glycans 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 and 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 (Sanver et al., 2016; Salas et al., 2011). Both narirutin (Fig. 3-5f) and hesperidin (Fig. 3-5g) 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. 3-5c). 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. 3-5b), rutin (Fig.

3-5c), taxifolin (Fig. 3-5e), narirutin (Fig. 3-5f) and eriodictyol (Fig. 3-5i). 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).

3.4 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 develop 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.

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Chapter 4: Removal of aflatoxin B_1 in aqueous solution by adsorption to corn by-products

Abstract: The contamination of food and feed with aflatoxin B_1 (AFB_1) causes serious health problems worldwide. Strategies are needed both to prevent aflatoxin production and reduce levels in contaminated products. In the present study, the adsorption of AFB_1 in aqueous solutions to natural fiber materials in powder form prepared from corn by-products was investigated. Powder made from corn cob (CCP) removed up to 98% of AFB_1 in 1h at 20°C, which was about 20 to 30% higher than corn bran powder (CBP). All determinants, including particle size, powder:AFB_1 ratio, adsorption time (0.25 to 24 h), temperature (4, 20, 37°C) and pH (2 to 8), had significant effects on % adsorption. The maximum % adsorption (97.99%) of CCP with the size ranging from 500-355 µm occurred at 20°C in 8 h, when the powder:AFB_1 ratio was 1:4 (mg:ng). The adsorption process followed a pseudo-second-order model, and fitted the Langmuir isotherm well. Besides, thermodynamic functions indicated that AFB_1 adsorbed onto CCP was exothermic. However, the typical cell wall components of corn (cellulose, hemicellulose, lignin) did not individually display strong adsorption behavior. In conclusion, CCP displays promising properties as an AFB_1 adsorbent that could be developed in further application of food detoxification.

Key words: Aflatoxin B_1 Corn cob Adsorption By-product Lignin

4.1 Introduction

Aflatoxin B_1 (AFB_1) is a toxic metabolite synthesized by *Aspergillus* sp. that accumulates in many foods and feeds, especially in cereal and cereal-based food (Marchese et al., 2018). Fungal growth occurs in a wide range of geographical areas characterized by warm and humid conditions

(Rasheed et al., 2020). Many strategies are employed to prevent fungal growth, and where this cannot be prevented, to decontaminate foods from mycotoxins. The strategies, including irradiation, treatment with neutral electrolyzed oxidizing water, microbial degradation, chemical synthetic material adsorption and biological material adsorption, have been found to be effective in reducing mycotoxin contamination (Rasheed et al., 2020). During the food storage and processing, some of these approaches can be efficient, eco-friendly or easily-operated, but may cause loss of nutrients and sensory properties, require specific equipment or induce inedible adsorbents to the system. Therefore, researchers are increasingly interested in mycotoxin detoxification approaches that are food grade, sustainable and widely sourced.

In recent years, some agricultural by-products have shown the capacity to adsorb up to 95% of aflatoxins in buffer at equilibrium. Examples include blueberry pomace (up to 78% for AFB_1, 72% for AFB_1, 71% for AFG_1, and 52% for AFG_2) (Rasheed et al., 2020), beetroot bagasse (13 to 55% for AFB_1), grape seed (around 20% for AFB_1), potato peels (10 to 45% for AFB_1) (Palade et al., 2020), shrimp shell (22 to 95% for AFM_1) (Assaf et al., 2018), banana peel (16 to 24% for AFB_1, 11 to 16% for AFB_1, 0 to 13% for AFG_1, and 8 to 40% for AFG_2) (Shar et al., 2016) and formosa firethorn leaves (Ramales-Valderrama et al., 2016). In a mixed mycotoxin solution (1 µg/mL of AFB_1, zearalenone (ZEN), ochratoxin A (OTA) and fumonisin B_1 (FB_1)), pomegranate peel (55%), plantain peel (67%), almond hull (87%), carobs (100%) and grape pomaces (94%) had the strongest adsorption ability with AFB_1, followed by ZEN; while the adsorption of pomegranate seed (86%), lemon residues (50 to 74%) and orange residues (51 to 61%) with ZEN was the strongest than other three mycotoxins (Greco et al., 2019). These fiber-rich agricultural by-products demonstrated high aflatoxins adsorption ability due to functional groups (e.g. hydroxyl, carbonyl, carboxylic/sulfhydryl carboxylic, phenolic, esters) presented in plant polysaccharides and lignin, and these groups might be the active sites for adsorption of mycotoxins (Rasheed et al., 2020).

Corn (*Zea mays*) is one of the most widely planted crops in the world. In 2019, the production of corn worldwide was about 1.15 billion tons. The major production regions are the Americas (49.20%), Asia (32.10%), Europe (11.60%) and Africa (7.10%) (FAOSTATS, 2021). As important by-products, about 18 kg of corn cob and 6 to 7 kg of corn bran can be generated for every 100 kg

corn grain (Rose et al., 2010; Wachirapakorn et al., 2016). Therefore, these large amount of corn by-products have been used as a feedstock for animal feed, production of biofuels, construction materials, absorbents for chemical waste, petrochemical industry, fermentation substrates and chemical carriers (Del Campo, 2010; Rose et al., 2010; Pointner et al., 2014). Corn cob or bran contain cellulose, hemicellulose and lignin, whereas the composition of polysaccharides varies greatly between species (Anderson and Clydesdale, 1980; Pointner et al., 2014). Cellulose is a common plant polysaccharide consisted of linear β-1,4-linked D-glucopyranose chains, and the chains link to each other by hydrogen bonds (Fig. 4-8d) (Dourado Fialho, 2015). The backbone of xylan is made up D-xylose linked by β-1,4 bonds, and arabinoxylan is the polymer where the xylan backbone is substituted with L-arabinose, D-galactose, glucuronic acid and ferulic acid (Fig. 4-8d) (de Souza, 2013). Lignin is made of three monomeric units of phenylpropanes (sinapyl alcohol, *p*-coumaryl alcohol, coniferyl alcohol) forming a complicated 3D structure. This structure is formed by different types of ether, ester bonds as well as C-C bonds (Dourado Fialho, 2015). The adsorption of various chemicals to carbonized or modified corn cob have been widely investigated. Corn cob (1.2 mg/mL) carbonized with H_2SO_4 was found to adsorb nearly 100% methylene blue in 1 h (Jawad et al., 2018). Similarly, H_3PO_4 modified corn cob (after crude polysaccharide extraction) adsorbed up to 97.50% of malachite green at the dose of 5 µg/mL (Hu et al., 2018). Biochar produced by pyrolysis of corn cob at 350°C and 450°C removed 8 to 73% of Pb^{2+} ions with different concentrations (0.25 to 3 mg/mL) of adsorbent dose (Assirey and Altamimi, 2021). However, no studies have focused on native corn cob.

To our best knowledge, there is no data about the reduction of AFB_1 by corn by-products through adsorption mechanism. The aim of this study was to investigate the feasibility of natural corn cob or bran as adsorbents to reduce AFB_1 content from aqueous solutions. Moreover, the adsorption mechanism was explored by the adsorption ability of individual cell wall components and model studies.

4.2 Materials and methods

4.2.1 Materials

All chemicals and solvents (acetonitrile, methanol, ethanol) used were analytical grade. Cellulose (Cotton linters, microcrystalline, 9004-34-6) was purchased from Sigma (UK). Xylan (Beechwood, >95%, xylose/glucuronic Acid=80.8/11.4, 9014-63-5) and arabinoxylan (Rye, high viscosity, ~90%, arabinose/xylose=38/62, 9040-27-1) were purchased from Megazyme (Australia). Lignin (HY-111830) was from MedChem Express (UK).

AFB_1 stock solution was prepared by dissolving 10 mg of AFB_1 (≥98%, Sigma) in 1 mL of dimethyl sulfoxide (DMSO), and then diluted to 5 mL with acetonitrile to give a stock solution with the concentration of 2 mg/mL, stored at -20°C.

4.2.2 Preparation of corn by-product powder

The fresh corn was purchased from local market (UK) in 2019. To obtain fresh corn bran, the internal content of corn seed was manually squeezed out after the seeds were removed from corn cob. 500g corn by-products (both corn bran and corn cob) were wet milled (Breville, Australia) with 1 L of cold extraction buffer (50 mM Tris and 100 mM Ethylene Diamine Tetraacetic Acid (EDTA) containing 2.63 mM sodium metabisulphite) for 5 min at 4°C. Next, the blended sample was filtered through four layers of cheesecloth. The residue fractions were washed with water three times and then with acetone three times. After freeze drying, the dried corn by-product powder was sieved through test sieves (Endecotts, UK) to divide the powder to 5 fractions: >750, 700-500, 500-355, 355-250 and <250 µm. The powders were named as prepared corn cob powder (CCP) and prepared corn bran powder (CBP) respectively.

The commercial CBP was obtained from local company (Dove's Farm, UK). It was dry milled for 5 min, and then was sieved as described above.

4.2.3 Batch adsorption experiments with corn by-products

AFB_1 solutions for all adsorption experiments were prepared by diluting the stock solution (2 mg/mL) with milli-Q water to the concentration of 200 ng/mL. The effects of different conditions

(corn by-product materials, powder particle sizes, powder:AFB_1 ratio, incubation temperature, incubation time, pH) were assayed. Activated charcoal (20-60 mesh) with AFB_1 solution (1:20, mg:ng), used as positive control (Diaz et al., 2002). It was placed on a 100 rpm of shaking water bath at 20°C for 1h. The 200 ng/mL of AFB_1 solutions were used as negative control. After incubation, the suspended CCP was removed by centrifugation at 12000 rpm for 10 min. The supernatants were collected for AFB_1 determination by HPLC.

The efficiency (%) of AFB_1 adsorption to corn by-products powder was calculated as equation (1):

$$\%\ \text{Adsorption}\ \left(\frac{C_0-C_t}{C_0}\right)\times 100\% \quad (1)$$

Where, C_t, the concentration of AFB_1 in the solution of sample groups after adsorption process, ng/mL; C_0, the initial concentration of AFB_1, ng/mL.

4.2.4 Model studies of AFB_1 adsorption

4.2.4.1 Adsorption kinetic study

The adsorption kinetics experiment was carried out by adding 3 mg of CCP to 300 µL of AFB_1 solution at concentration of 60 µg/mL. The mixture was placed on the shaking water bath (100 rpm) at 20°C. After centrifugation, the supernatant containing residual AFB_1 was measured from 5 min to 24 h with HPLC.

The adsorption capacity of CCP for AFB_1 was calculated as equation (2) (Ji and Xie, 2021):

$$q_e = \frac{(C_0-C_e)V}{m} \quad (2)$$

Where, q_e, the adsorption amount of AFB_1 at equilibrium condition, µg/mg; C_0, the initial concentration of AFB_1, µg/mL; C_e, the equilibrium concentration of AFB_1 after adsorption process, µg/mL; V, the volume of AFB_1 solution, mL; m, the weight of CCP, mg.

The experimental data from present study could be fitted to two classic kinetic models, pseudo-first-order (3) and pseudo-second-order (4):

$$ln\frac{q_e-q_t}{q_e} = -k_1 t \quad (3)$$

$$\frac{t}{q_t} = \frac{1}{k_2 q_e^2} + \frac{t}{q_e} \quad (4)$$

Where, t, the adsorption time, min; q_t, the adsorption amount of AFB_1 at time point t, μg/mg; k_1, the rate constant of pseudo-first-order, 1/min; k_2, the rate constant of pseudo-second-order, mg/(μg·min). The linearized plots for pseudo-first-order and pseudo-second-order model were estimated using the slope and intercept of $\ln(q_e-q_t)$ vs. t and (t/q_t) vs. t respectively.

Moreover, the experimental data could be calculated as the intraparticle diffusion model (5) (Ma et al., 2021):

$$q_t = k_{id}t^{0.5} + c \quad (5)$$

Where, k_{id}, the rate constant of intraparticle diffusion model, μg/(mg·$min^{-0.5}$); C, to reflect the thickness of the boundary layer, μg/mg. The plot for the intraparticle diffusion model was estimated using the slope and intercept of q_t vs. t. The intercept of the plot reflects the thickness of the boundary layer.

4.2.4.2 Adsorption isotherm models

The adsorption isotherm experiment was performed at 20°C by adding 3 mg of CCP to 300 μL of AFB_1 solution at various initial concentrations from 0.1 to 90 μg/mL. The mixture was placed on the shaking water bath (100 rpm) immediately until adsorption equilibrium.

The adsorption isotherms data could be conducted using Langmuir (6) and Freundlich (7) models (Avantaggiato et al., 2013):

The Langmuir model is presented as $q_e = \frac{q_m k_L C_e}{1+k_L C_e}$ (6)

Where, k_L, the Langmuir constant, mL/μg; q_m, the maximum adsorption capacity, μg/mg. The parameters for the Langmuir model were estimated by plotting C_e/q_e vs. C_e.

The Freundlich model is presented as $q_e = k_F C_e^{\frac{1}{n}}$ (7)

Where, k_F, the Freundlich constant, mL/μg; 1/n, the degree of heterogeneity of adsorbent surface. The parameters for the Freundlich model were estimated by plotting $\ln q_e$ vs. $\ln C_e$

4.2.4.3 Adsorption thermodynamic study

The adsorption thermodynamic experiment was carried out by adding 3 mg of CCP to 300 μL of

AFB_1 solution at concentration of 60 µg/mL. The mixture was placed on the shaking water bath (100 rpm) immediately at various temperature (20 to 50°C) until adsorption equilibrium.

The thermodynamic constants were calculated by the van't Hoff equations (8)-(11). The constants were the Gibbs free energy (ΔG^0), standard entropy change (ΔS^0) and standard enthalpy change (ΔH^0) (Ji and Xie, 2021).

$$K_T = \frac{q_e}{C_e} \quad (8)$$

$$\Delta G^0 = -RTlnK_T \quad (9)$$

$$\Delta G^0 = \Delta H^0 - T\Delta S^0 \quad (10)$$

$$lnK_T = \frac{\Delta S^0}{R} - \frac{\Delta H^0}{RT} \quad (11)$$

Where, K_T, the equilibrium constant, (mL/mg); ΔG^0, the Gibbs free energy of adsorption, (kJ/mol); R, general gas constant 8.314 J/(mol·K); T, the adsorption absolute temperature, (K); ΔH^0, the enthalpy change, kJ/mol; ΔS^0, the entropy change, J/(K·mol). The plot for the van't Hoff equations was estimated using the slope and intercept of lnK_T vs. 1/T, and the slope and intercept approximately equaled to $-\Delta H^0/R$ and $\Delta S^0/R$ respectively.

4.2.5 Adsorption stability of CCP-AFB_1 complex after several washes

According to Assaf et al. (2018), following the adsorption experiment with CCP (500-355 µm) as previously described, the adsorption stability was focused the CCP-AFB_1 complex. 20 mg of CCP was added in 2 mL of 200 ng/mL AFB_1 solution and then incubated at 20°C for 1 h. Next, the mixture was poured to filtration column (15mL, TELOS, US) for washing steps (Fig. 4-1). The complex was washed with 2 mL of water or ethanol for 5 times each, and every wash took 10 min. The washed AFB_1 content was quantified by HPLC.

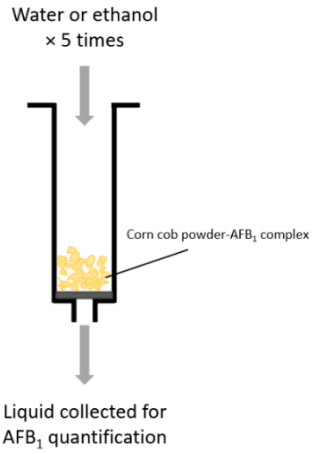


Fig. 4-1 The schematic diagram of washing CCP-AFB_1 complex with water or ethanol.

4.2.6 Adsorption experiment with cell wall components

The most abundant components of corn cob are cellulose, lignin, arabinoxylan and xylan (Fig. 4-5b). commercial samples of these were used in the adsorption experiment following the same binding experiments as described for CCP and CBP. 10 mg of each standard was incubated with 1 mL of 200 ng/mL AFB_1 solution at 20°C for 1 h. Tube was then centrifuged at 12000 rpm for 10 min. The supernatant was collected for AFB_1 content detection by HPLC, and the % adsorption was calculated as described before.

4.2.7 AFB_1 quantification

Residual AFB_1 identification was carried out according to the report by Ma et al. (2021) with some modification. The measurement was performed by a Shimadzu (Japan) high performance liquid chromatography (HPLC) system coupled with fluorescence detector (RF-20A xs). The AFB_1 samples (50 µL) were injected to a phenomenex Luna C18 (5µm particle size, 150×4.6mm) operated at 40°C. The mobile phase was composed of HPLC grade water: acetonitrile: methanol (70:15:15/vol:vol:vol), and its flow rate was 1 mL/min. Fluorescence detection was used with an excitation and emission wavelengths of 360 and 440 nm respectively. The retention time for AFB_1 was around 23.1 min. A calibration curve was established using different concentrations of AFB_1 standard ranging from 3.125 to 200 ng/mL (R^2=0.9997). The limit of detection was 0.1 ng/mL.

4.2.8 Statistical analysis

All results were shown as the mean value ± standard deviation of triplicate experiments. Data were analyzed using GraphPad 7 by one-way ANOVA and Tukey's multiple range tests. Model fitting was performed by Origin2018. P values < 0.05 were considered significant.

4.3 Results and discussion

4.3.1 Effect of factors on AFB_1 adsorption to corn by-products

The % adsorption of AFB_1 by corn by-products in aqueous solution was studies. This was simultaneously assessed as a function of waste materials, powder particle sizes, powder:AFB_1 ratios, incubation temperatures, incubation times and pH.

To screen the best corn by-product for AFB_1 adsorption, batch adsorption experiments were performed with prepared CBP, prepared CCP and commercial CBP with constant conditions. As shown in Fig. 4-3a, apart from particle sizes >750 µm, the % adsorption of prepared CCP (79.54% to 90.02%) was consistently higher than that of prepared CBP (64.52 to 66.13%) and commercial CBP (39.20% to 47.33%). Corn bran is characterized by a porous pericarp layer with a thickness of 100 to 250 µm (Roye et al., 2019). Nevertheless, corn cob generally contains an inner pith layer of a closed cellular structure type, a middle wood ring layer of cross-interconnected sponge-like pores and the outer glume layer of squeezed bran (Fig. 4-2) (Zou et al., 2021). Thus, the CCP containing three types of structure showed a higher adsorption ability than single layer of CBP.

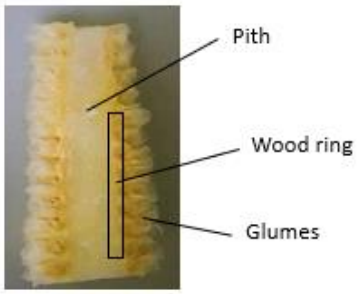


Fig. 4-2 The cross-section of corn cob.

From the perspective of particle size (Fig. 4-3a), the % adsorption of all three adsorbents was significantly affected by particle size (p<0.005). For CCP, compare to the smallest size (<250 µm), when the size was larger than 500 µm, the % adsorption significantly decreased to 56.12% (p<0.005), whereas the powder with size smaller than 500 µm showed no significant effect on % adsorption (86.49 to 90.03%) (p>0.05). Similar trend could be found in mycotoxin adsorption with grape pomace particle (Avantaggiato et al., 2013). This might be because excessive particle size, such as >500 µm of CCP and grape pomace particle (Avantaggiato et al., 2013), caused the

reduction of accessibility of active sites to mycotoxins. As the CCP showed the best effect on AFB_1 adsorption, it was selected as the adsorbent for further studies.

Batch adsorption experiments with different powder:AFB_1 ratios were tested (p<0.0001) (Fig. 4-3b). The result displayed that the % adsorption continuously raised from 23.16 to 97.99% by increasing ratios from 1:200 (1 mg/mL for cob powder dose) to 1:4 (50 mg/mL for cob powder dose). This indicated that higher ratio/dose provided more active sites resulting in larger % adsorption. Similar results could be found in other AFB_1 adsorption materials. For example, the adsorption capability of blueberry pomace was 16 to 79% observed by increasing dosage from 0.5 to 3.0 mg/mL (Rasheed et al., 2020). The toxin removal of a synthetic magnetic adsorbent, Fe_3O_4@ATP, significantly improved (54 to 87%) with increase in the dosage from 0.5 to 3 mg/g peanut oil, and then the removal ability approaches constant from 0.3 to 1.0% (Ji and Xie, 2021). Likewise, *Pleurotus eryngii* mycelium in the dosage of 25 to 87.5 mg/mL could adsorb with AFB_1 from 7.5 to 60%, and the adsorption kept stabilized from 100 to 200 mg/mL (Haidukowski et al., 2019). Somewhat differently, modified organo-rectorites (ORts) as AFB_1 adsorbent showed an increasing adsorption (65 to 89%) with the dosage ranging from 0.5 to 2.0 CEC (cation exchange capacity), while the % adsorption decreased to 82.00% at the dosage of 2.5 CEC. This was because large dosage (2.5 CEC) of modifier molecules could aggregate together and congregate on the ORts surface, which reduced the proportion of active ingredients in the adsorbent (Sun et al., 2018).

The dependence of incubation times on % AFB_1 adsorption was investigated (p<0.0001) (Fig. 4-3c). Over 80% of the total adsorption occurred in first 15 min. With the increase of incubation time, the adsorption efficiency increased gradually up to 93.46%, and this maximum adsorption was reached at 8 h. Then the % adsorption kept stable in next 16 h. This might be because a large number of active adsorption sites existed on the cob powder increasingly adsorbed AFB_1 molecules at beginning, while slowed down with time due to the decreased sites and finally reached adsorption equilibrium after 8 h (Rasheed et al., 2020). Guo et al. (2012) reported that the adsorption of commercial yeast powder and patulin reached to highest point (about 100%) at 30 h, while that of lab-prepared yeast powder was 6 h later (about 100%). Palade et al. (2020) found AFB_1 adsorption occurred rapidly (<5 min) by the food or agricultural by-products (carrot,

celery, granny apple, red potato, white potato and sea buckthorn) accompanied by the increase until the 200 min threshold; similarly, the adsorption of ZEN could be seen at less than 5 min and its threshold time was after 90 min. Avantaggiato et al. (2013) demonstrated that around half of mycotoxins (AFB_1, ZEN, OTA and FB_1) in a mixed solution was adsorbed by grape pomace in less than 3 min, and the maximum adsorption occurred in 15 min. Therefore, it can be seen that the adsorption between mycotoxins and adsorbents happened rapidly, and a predominant amount of total adsorbed mycotoxins would be adsorbed in the early stage. Once the adsorption was stable, the free adsorbates (toxins) in the solution would not significantly decrease.

The AFB_1 adsorption experiments were subsequently conducted at different temperatures, 4°C, 20°C, biological temperature (37°C), warm temperature (50°C) and boiling temperature (100°C) for 1 h (p<0.0001). Fig. 4-3d shows that, apart from 100°C (decreased to 71.74%) (p<0.0001), the adsorption of AFB_1 over cob powder was independent of temperature, with binding of 84.18% at 4°C, 84.47% at 20°C, 84.96% at 37°C and 86.97% at 50°C (p>0.05). Therefore, CCP could be used in a wide range of temperatures used in food processing for AFB_1 adsorption/removal. The effect of temperatures on AFB_1 adsorption was also investigated by previous studies. The by-products of grape seed (approximately 45%) and sea buckthorn (approximately 35%) did not significantly change the AFB_1 adsorption ability at 25°C, 37°C and 40°C (Palade et al., 2020). However, temperature significantly affected adsorption behavior on two novel materials. Fe_3O_4@ATP adsorbed 83% of AFB_1 from peanut oils at 40°C, and the % adsorption increased 5.85% with the increasing temperature to 60°C, while fell back to 82.02% when the temperature continuously raised to 110°C (Ji and Xie, 2021). Besides, the AFB_1 adsorption of mesoporous silica prepared from rice husk kept stable (around 95%) in oil matrix between 10°C to 20°C, but decreased sharply to 75% when the temperature increased to 30°C (Li et al., 2020). These studies indicated that the effect of temperature on adsorption varied from materials.

To evaluate the % AFB_1 adsorption as a function of pH, batch adsorption tests were performed with pH ranging from 2 to 8 (Fig. 4-3e). The result revealed that pH of adsorption solutions had significant impact on the adsorption (83.81 to 87.18%) of AFB_1 to CCP (p<0.05). However, compare to pH 7, most of pH conditions did not significant affected adsorption (p>0.05). PH is a key factor impacting the surface charge of adsorbents and the ionization level of toxins (Zahoor

and Ali Khan, 2014). In general, the adsorption ability of toxins varying surface charges would be greatly affected by pH, such as malachite green adsorbed by acidified corn cob (Hu et al., 2018), ZEN, OTA and FB_1 adsorbed by grape pomace (Avantaggiato et al., 2013), and methylene blue adsorbed by carbonized corn cob (Jawad et al., 2018). However, there was no ionization on AFB_1 molecule (Fig. 4-3f) under different pH conditions (Sun et al., 2018). Therefore, not only the % adsorption of CCP on AFB_1 was not significantly affected by pH at range from 2 to 8 in present study, but also in the adsorption of iron oxide carbon nanocomposites (prepared from bagasse) (Zahoor and Ali Khan, 2014), blueberry pomace (Rasheed et al., 2020) and grape pomace (Avantaggiato et al., 2013). This result indicated that the CCP can be considered as an AFB_1 adsorbent in liquid foods under most of pH conditions.

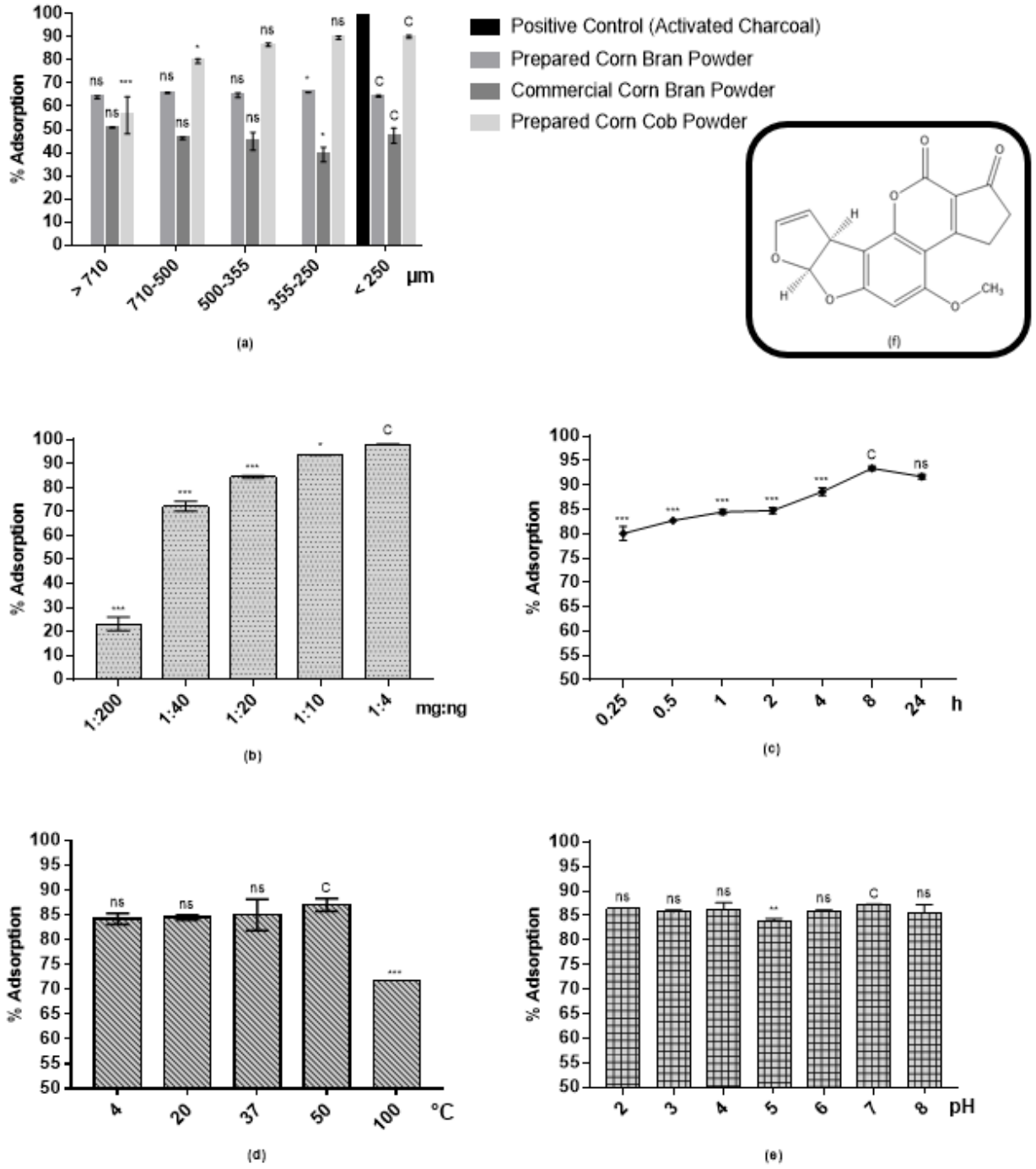


Fig. 4-3 % Adsorption of CBP and CCP to AFB_1 affected by particle sizes (a), powder:AFB_1 ratios (b), incubation times (c), incubation temperatures (d) and pH (e), and chemical structure (f) of AFB_1. * represents significant differences compare treated groups under different conditions to the group (C) showing the highest AFB_1 % adsorption. ***, $p \leqslant 0.0001$; **, $p \leqslant 0.001$; *, $p \leqslant 0.05$; and ns, $p > 0.05$. Results are means of three replicates.

4.3.2 Model studies of AFB_1 adsorption

4.3.2.1 Adsorption kinetic study

Kinetic study of adsorption describes reaction rate of the process and adsorption capability of the adsorbents (Rasheed et al., 2020). Fig. 4-4a illustrated that AFB_1 adsorption capacity of CCP raised rapidly within 5 h caused by the strong capillary force formed from microporous and mesoporous material (Ma et al., 2021). Then the equilibrium was achieved in 8 h and there was no noticeable change in adsorption after this time point. To analysis the controlling mechanism of adsorption process, two classic models (pseudo-first-order and pseudo-second-order) were applied. The fit of the pseudo-first-order (eq. 3) to AFB_1 adsorption data is shown in Fig. 4-4b and the pseudo-second-order (eq. 4) in Fig. 4-4c, and the kinetic parameters are listed in Table. 4-1. According to the result, the pseudo-second-order model (R^2=0.9979) better fitted than the pseudo-first-order model (R^2=0.7727). Furthermore, the experimental adsorption capacity (2.9910 μg/mg) was closer to the calculated adsorption capacity (3.0533 μg/mg) by pseudo-second-order model than pseudo-first-order model. This suggests the AFB_1 adsorption process involves chemisorption (Ma et al., 2021; Ji and Xie, 2021).

In general, the near-adsorbent adsorption process involves three steps, which are 1) boundary layer diffusion – adsorbate diffusion across the liquid film surrounding the adsorbent; then 2) intraparticle diffusion: further diffusion within internal pores of adsorbent; finally 3) adsorption of adsorbate on the surface of adsorbent (Ma et al., 2021). Moreover, the boundary layer and/or intraparticle diffusion is the controlling step, and the last step is relatively fast in the majority of adsorption systems (Ma et al., 2021). Therefore, the intraparticle diffusion model (eq. 5) is usually conducted to fit the experiment result for the explanation of the diffusion mechanism. The intraparticle diffusion plot (Fig. 4-4d) displayed two-linearity in the adsorption of AFB_1 on CCP, which indicated that the adsorption process involved the diffusion of AFB_1 from solution to the boundary layer of cob powder and the intraparticle diffusion into the porous structure of cob powder at low concentration of AFB_1 to final equilibrium (Cai et al., 2019; Ma et al., 2021). And the plot did not pass through the origin, so the boundary layer diffusion and intraparticle diffusion controlled the rate-controlling step (Ma et al., 2021). It is reported that the meso- and macropores

morphology presents an easy accessibility of adsorbates into the internal structure of the adsorbents, while the micropores are narrow for adsorbates to access the adsorbents so that limit the inner diffusion rate of adsorption (Ma et al., 2021). Therefore, the cob powder might have a large portion of meso- and macropores, while have small portion of micropores (Ma et al., 2021). In addition, intercept of the intraparticle diffusion plot (C) suggested the thickness of the boundary layer (Table. 4-1), reflecting the contribution of boundary layer in adsorbate adsorption rate (Ma et al., 2021).

Table. 4-1 Kinetic parameters of pseudo-first-order, pseudo-second-order and intraparticle diffusion for the adsorption of AFB_1 on CCP (20°C)

	$q_{e,exp}$ (μg/mg)		$q_{e,cal}$ (μg/mg)	R^2
Pseudo-first-order	2.9910	k_1 (1/h) -0.0071	1.5746	0.7727
Pseudo-second-order		k_2 (mg/(μg·h)) 0.37075	3.0533	0.9979
Intraparticle diffusion	-	k_{id} (μg /(mg·$h^{0.5}$)) 0.5852	C 0.7247	0.8386

$q_{e,exp}$, the experimental adsorption capacity; $q_{e,cal}$, the calculated adsorption capacity.

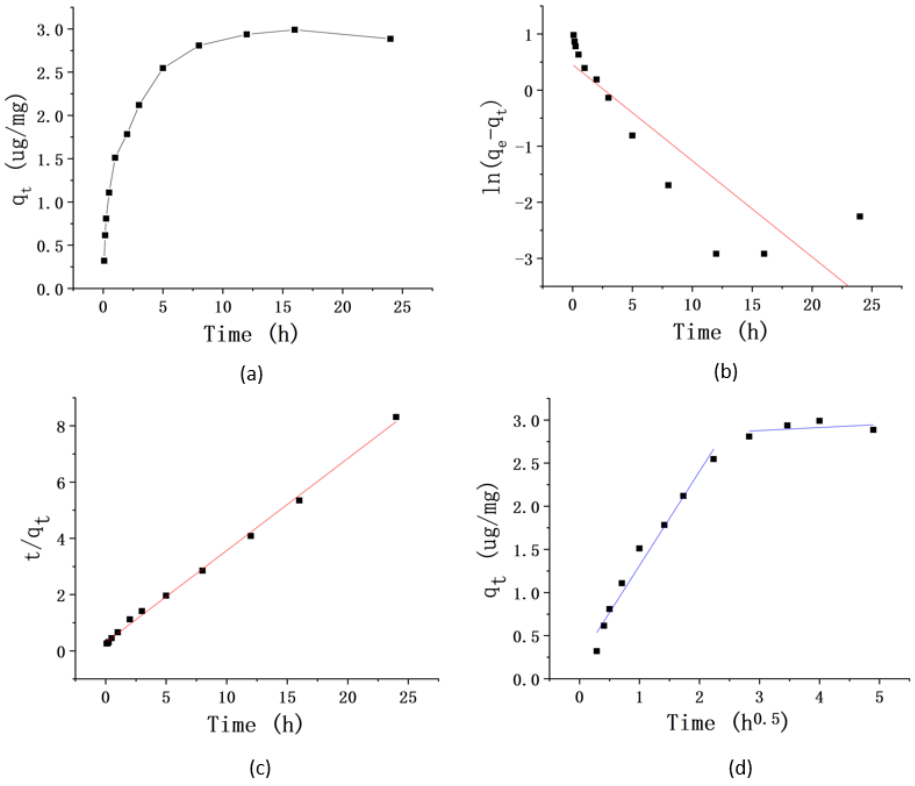


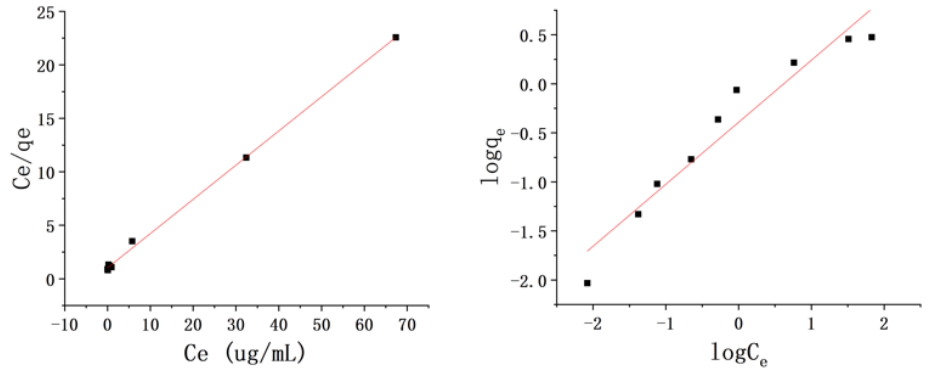
Fig. 4-4 Kinetics study of the adsorption of AFB_1 on CCP (a), and fitting of data with pseudo-first-order model (b), pseudo-second-order model (c) and intraparticle diffusion model (d).

4.3.2.2 Adsorption isotherm models

Adsorption isotherm models are applied to describe the maximum adsorption capability of AFB_1 adsorbed on CCP and molecules under different initial concentration at constant temperature (Rasheed et al., 2020). Two non-linear isotherm models were used to investigate the adsorption characteristic: Langmuir (eq. 6) and Freundlich (eq. 7) isotherm models. Langmuir model is one of the most classic models describing an ideal adsorption with the characteristics of monolayer, no interaction between adsorbates and uniqueness of all adsorption sites on the adsorbent surface (Ma et al., 2021). Whereas, Freundlich model is the earliest isotherm used to describe the adsorption performance on a heterogeneous surface, no restricted to the formation of monolayer (Chen et al., 2016; Avantaggiato et al., 2013). The linear regression fitting figures of two models could be found in Fig. 4-5a-b, and the parameters were listed in Table. 4-2. From the result of present study, the Langmuir isotherm demonstrated a better fit with the experimental data (R^2=0.9986) than Freundlich (R^2=0.9344) model. This illustrated that the isothermal adsorption by CCP could be better described by the AFB_1 adsorption on homogeneous CCP surface and no further adsorption occurs once AFB_1 molecule adsorbed at specific sites on cob powder (Sun et al., 2018). According to the Langmuir model, the maximum adsorption capability of CCP was calculated to be 3.1268 μg/mg. This AFB_1 adsorption capability was far higher than banana peel (0.0084 μg/mg) (Shar et al., 2016), but similar to grape pomace (2.8600 μg/mg) (Avantaggiato et al., 2013) and blueberry pomace (3.2290 μg/mg) (Rasheed et al., 2020). Moreover, k_L is a constant related to the energy and affinity of CCP, which indicated the adsorption was favourable when k_L above 0 (Ma et al., 2021).

Table. 4-2 Isotherm model parameters for the adsorption of AFB_1 on CCP (20°C)

			R^2
Langmuir model	**q_m (μg/mg)**	**k_L (mL/μg)**	
	3.1268	0.3109	0.9986
Freundlich model	**1/n**	**k_F (mL/μg)**	
	0.6325	0.4057	0.9344



(a) (b)

Fig. 4-5 Langmuir plot (a) and Freundlich plot (b) for the adsorption of AFB_1 on CCP

4.3.2.3 Adsorption thermodynamics study

Adsorption thermodynamics study can also provide theoretical evidence of feasibility and spontaneity of adsorption reaction (Rasheed et al., 2020). The van't Hoff plot and thermodynamics parameters (eq. 8-11) for the adsorption of AFB_1 on CCP was demonstrated in Fig. 4-6 and Table. 4-3 respectively. For this AFB_1 adsorption, the equilibrium constant K_T decreased with temperature increasing, which means the experimental and theoretical adsorption capacities declined with the increased temperature, as well as the absolute Gibbs free energy change (ΔG^0) rose with the increased temperature. This indicated that higher temperature lowered the adsorption driving force (Ji and Xie, 2021). However, the positive ΔG^0 at all temperatures suggested the adsorption process occurred non-spontaneously at certain temperature (Avantaggiato et al., 2013). Furthermore, the enthalpy change (ΔH^0) in this adsorption process was negative confirming the reaction was exothermic. The magnitude of value ΔH^0 can also describe the type of adsorption (either physical or chemical adsorption). Generally, physical adsorption is rapid and weak, and the interactions include hydrogen bonds, van der Waals, dipole-dipole, and induced dipole; while chemical adsorption is highly specific and slow, as well as the interaction exists electron transfer between molecules, e.g. covalent bonds (Karakaya, 2011; Avantaggiato et al., 2013). When ΔH^0 <20 kJ/mol, the adsorption is considered as physisorption, whereas the reaction is chemisorption (Avantaggiato et al., 2013). In the adsorption of AFB_1 on CCP, the ΔH^0 was -19.1060kJ/mol, which suggested a physical adsorption. In the kinetic study, it was suggested that a chemical adsorption was involved in the AFB_1

adsorption with CCP. Thus, both chemical and physical adsorption might exist in this process (Hu et al., 2018). In the solid-liquid system, a natural adsorption of adsorbate onto adsorption surface would lower the chaos of system, which is represented by the entropy change (ΔS^0) below 0 (Hu et al., 2018). In the present work, the ΔS^0 was found to be -0.0824 kJ/(K·mol), illustrating the randomness of AFB_1 molecules on the CCP interface reduced with the increasing temperature (Rasheed et al., 2020). It is believed that this phenomenon corresponds to polar noncovalent interactions (Avantaggiato et al., 2013). In a word, in terms of the thermodynamics parameters, the adsorption process of AFB_1 on CCP was an exothermic and chaos reduced adsorption interaction.

Table. 4-3 Thermodynamics parameters for the adsorption of AFB_1 on CCP

Temperature (K)	K_T	$\ln K_T$	ΔG^0 (kJ/mol)	ΔH^0 (kJ/mol)	ΔS^0 (kJ/(K·mol))	R^2
293	0.1195	-2.1249	5.0460	-19.1060	-0.0824	0.9589
303	0.1069	-2.2363	5.8703			
313	0.0744	-2.5987	6.6946			
323	0.0598	-2.8163	7.5189			

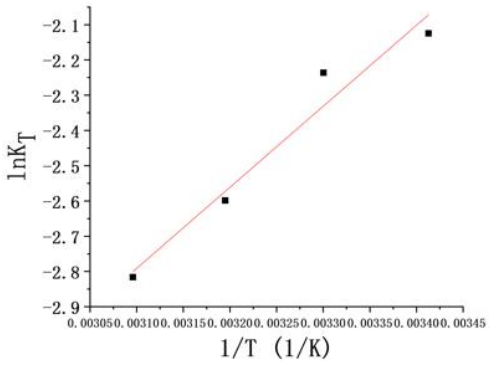


Fig. 4-6 Vant Hoff's plot for the adsorption of AFB_1 on CCP

4.3.3 Adsorption stability after successive washes in water or ethanol adsorbed with AFB_1

After 1 h incubation of CCP with AFB_1, the adsorption stability of the complex was assessed by quantifying AFB_1 in each washing liquid. As shown in Fig. 4-7, AFB_1 was released from the complex after washes by either water or ethanol. In first two washes of water, 10.42% and 8.80% of total AFB_1 (200 ng/mL) was washed off respectively, and around 6 to 7% of that was released in the rest of each successive washes. After five-time wash, around 46% of total adsorbed AFB_1 (168.94

ng) was eluted. Conversely, ethanol removed 67.68% of total AFB_1 (200 ng) from the complex in the first wash, and AFB_1 was desorbed from 10.26 to 1.33% during rest four washes. Thus, nearly all the adsorbed AFB_1 was released from CCP. The reversibility indicated that this non-specific and weak adsorption formed non-covalent bond between CCP and AFB_1 could be disrupted after successive washes (Karakaya, 2011; Assaf et al., 2018). As AFB_1 is soluble in ethanol, while sparingly soluble in water, the adsorption was more unstable by the disruption of ethanol than water. Therefore, CCP as the adsorbent to remove AFB_1 in food matrixes should be replaced once the adsorption reaches equilibrium. By contrast, AFB_1 could be easily collected from the complex on the basis of its stability.

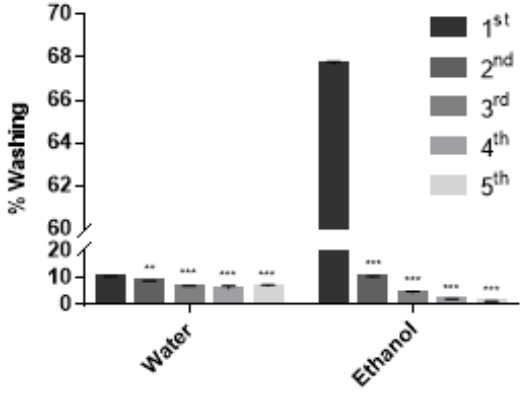


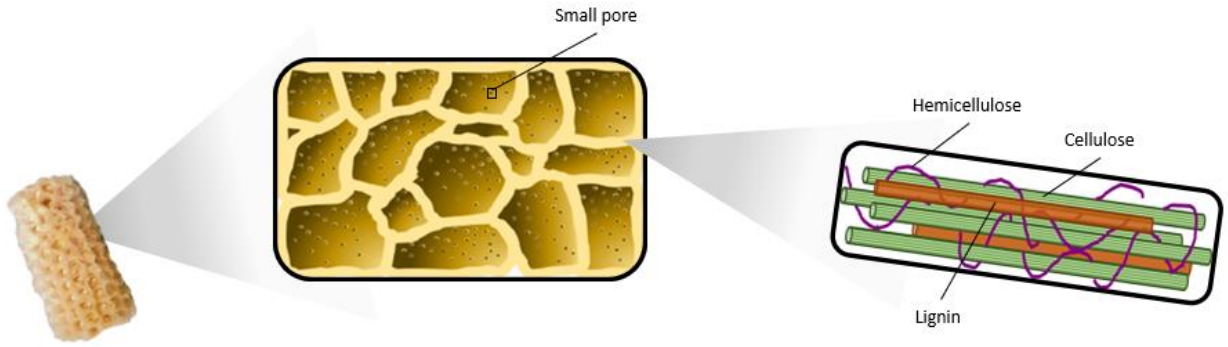
Fig. 4-7 Effect of successive washes on CCP-AFB_1 complex adsorption stability. Adsorption was determined after incubation of AFB_1 (200 ng/mL) with CCP (500-355 μm) for 1 h at 20°C. The complex was then washed five times with water or ethanol. * represents significant differences compare each following washes to first wash. ***, $p \leqslant 0.0001$; **, $p \leqslant 0.001$; *, $p \leqslant 0.05$ and ns, $p > 0.05$. Results are means of three replicated dishes.

4.3.4 AFB_1 adsorption of corn cob components

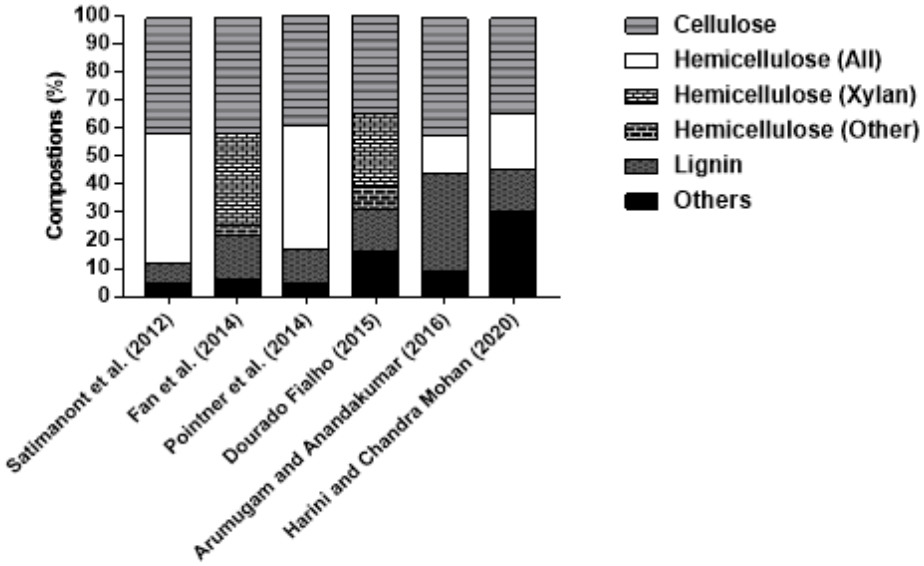
With an observation by scanning electron microscope, the micrograph demonstrated that the corn cob has an ordered cell wall with small pores forming a honey-comb structure (Genovese et al., 2015). The cell wall was mainly made of cellulose, cross-linked with hemicellulose and lignin (Fig. 4-8a). In terms of previous literatures, the cellulose, lignin and hemicellulose account for up to 42%, 35% and 46% respectively. Among the hemicellulose, xylan is found to take up 26 to 33% of total dry weight (Fig. 4-8b), which is the most abound hemicellulose in cob cell wall. When xylan units link with arabinose (2.4 to 3.6% in corn cob) as side groups, the polysaccharides are identified as arabinoxylan (Silva et al., 2015; Serra et al., 2020). Thus, commercial cellulose, lignin,

xylan and arabinoxylan were used for AFB_1 adsorption efficiency investigation.

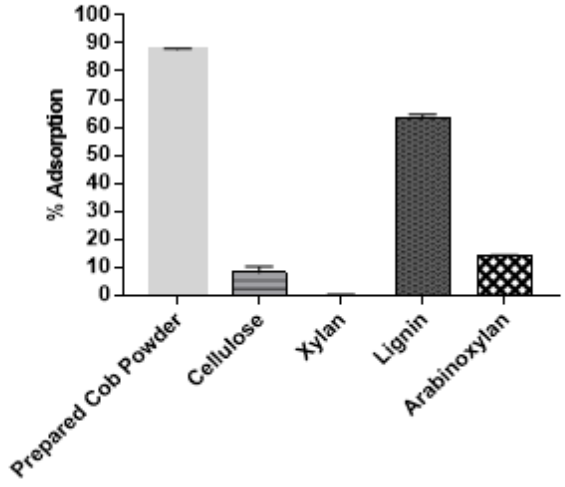
These isolated compositions all had various degrees of lower AFB_1 adsorption capability compared to CCP at same dose (Fig. 4-8c). There was little adsorption of AFB_1 with xylan, while cellulose and arabinoxylan showed low adsorption at 8.16% and 14.03% respectively. However, lignin displayed surprisingly high (63.43%) adsorption ability. According to the chemical structure of each component (Fig. 4-8d and e), the AFB_1 adsorption might be the hydrophobic effect between linked monomers in the cob components and AFB_1 molecule, such as the ferulic acid of arabinoxylan and phenylpropanes of lignin. However, even at same amount, the % adsorption of any single cob component was far less than the natural cob powder. Considering that each component accounted for a portion of the total weight of corn cob, the role of individual components in adsorption might be very limited. Therefore, the adsorption may not only depend on the interaction between AFB_1 with cell wall components, but also a significant effect of cob cellular organization on adsorption properties.


Small pore
Hemicellulose
Cellulose
Lignin

(a)


Compostions (%)
100
90
80
70
60
50
40
30
20
10
0
Satimanont et al. (2012)
Fan et al. (2014)
Pointner et al. (2014)
Dourado Fialho (2015)
Arumugam and Anandakumar (2016)
Harini and Chandra Mohan (2020)
Cellulose
Hemicellulose (All)
Hemicellulose (Xylan)
Hemicellulose (Other)
Lignin
Others

(b)


% Adsorption
100
90
80
70
60
50
40
30
20
10
0
Prepared Cob Powder
Cellulose
Xylan
Lignin
Arabinoxylan

(c)

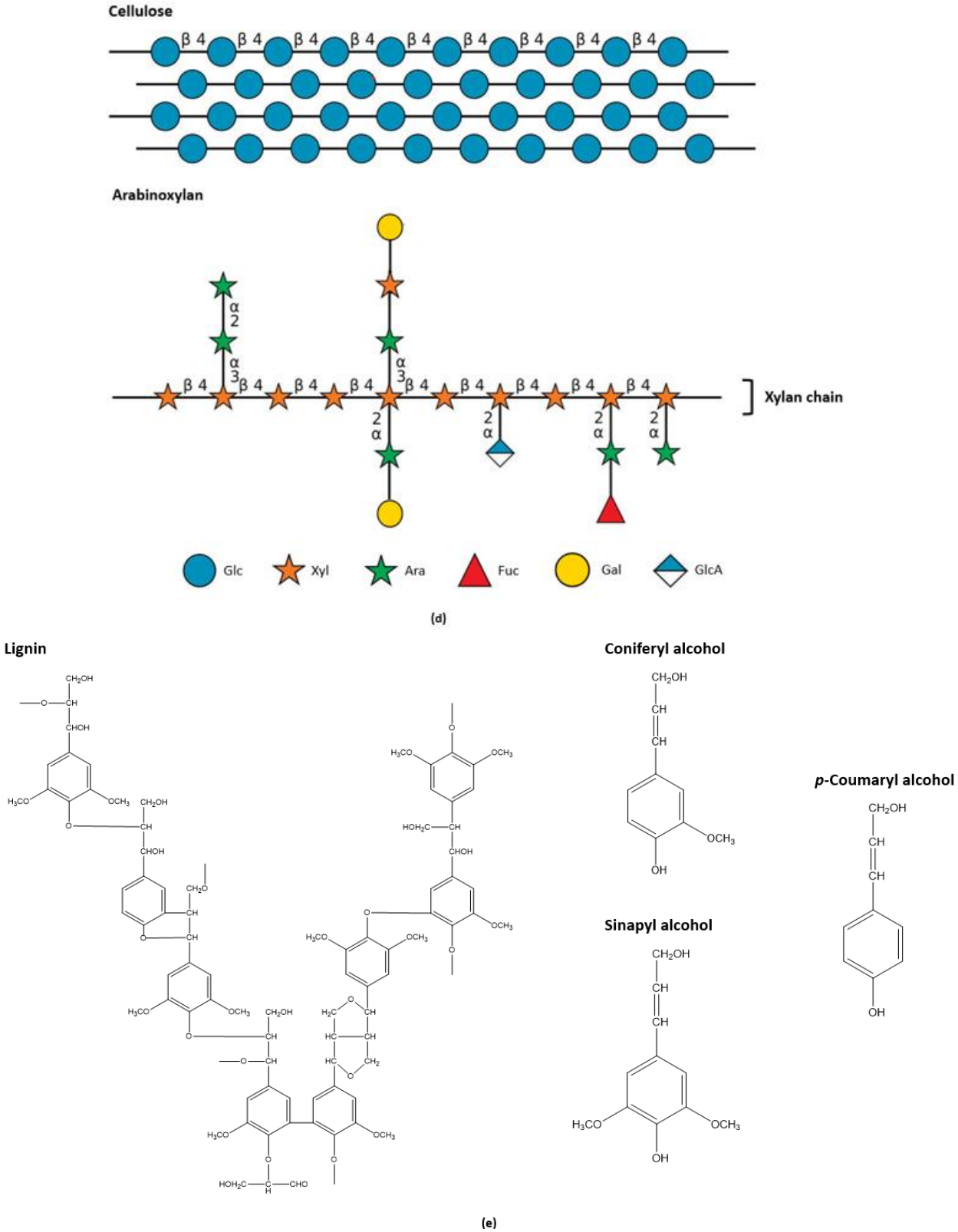


Fig. 4-8 Structural organization of corn cob (Genovese et al., 2015) (a), chemical compositions of corn cob in the literature (b), % adsorption of cob compositions (10 mg) to AFB_1 (200 ng/mL) for 1 h at 20 °C (c), possible schematic of structures for cellulose and (arabino)xylan (de Souza, 2013; Neelamegham et al., 2019) (d) and schematic formular of the lignin (angiosperm) structure, and chemical structures for coniferyl alcohol, sinapyl alcohol and p-coumaryl alcohol (Moore et al., 2011) (e).

4.4 Conclusion

The present work provided evidence that powdered corn by-products, corn cob in particular, could be used as a low-cost and non-toxic material for AFB_1 removal from aqueous solution. The finding showed that, within one hour, the CCP had high % adsorption of up to 98%, and it was significantly affected by powder:AFB_1 ratio, adsorption duration (0.25 to 8 h), particle size (<500 μm), temperature (4, 20, 37 and 50°C) and pH (2 to 8). The investigation of theoretical adsorption model was conducted by the adsorption kinetics, thermodynamics and isotherms models, which suggested that the AFB_1 adsorption on CCP was a fast and exothermic reaction, followed with a homogeneous adsorption process. The adsorption was reversible, and could be easily desorbed by ethanol. For the first time, AFB_1 adsorption by individual cob components (cellulose, (arabino)xylan, lignin) was investigated in the present study. The result demonstrated the adsorption of AFB_1 on CCP not only replied on the interaction between AFB_1 molecules and cob components, but the spatial cellular organization of corn cob played the most important role.

In conclusion, CCP can be a potential toxin removal material that is applied to decontaminate AFB_1 from liquid. However, the present study is primary research for corn cob as an AFB_1 adsorbent in an ideal environment (e.g. aqueous solution). For further studies, the adsorption capacity should be investigated in various liquid food matrixes, e. g. oil and cereal milk. It is also interesting to understand the adsorption capacity of CCP for other mycotoxins, so that this adsorbent would be used in mycotoxin removal in a real food scenario (e. g. fruit juice and spice extracts). Furthermore, in the practical application, the competitive adsorption of different compounds on CCP should be considered as well.

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Chapter 5: General Discussion and Conclusion

5.1 The outcomes of this thesis

The worldwide contamination of human foods and animal feeds with mycotoxins is a persistent problem. It brings serious health threats to human and animals, and enormous economic losses in food industry and animal husbandry every year (Wang et al., 2018). Therefore, the approaches to reduce or eliminate the contamination of mycotoxins and their producer, fungi, remain a research priority. Many mitigation approaches have been explored in recent decades, but not one approach has been found to be 100% for prevention of contamination in all food matrixes. But the development of technologies always tends to be low toxicity, cost-effective, easy operated, efficient and environmental friendly. And this is also the aim of this thesis.

5.1.1 Comprehensive review of mitigation of fungi and mycotoxins

- This work provided a comprehensive overview of the main technologies in the decontamination of both fungi and mycotoxins in recent decades. These technologies were classified to physical, chemical and biological approaches according to if extra chemicals/biological materials were induced to the system.

- Among the decontamination approaches, conventional elimination approaches (e.g. temperature and humidity control, cleaning, milling and sorting, chemical antifungal/anti-mycotoxins agents) have been gradually applied in large scale storage and production, while the emerging approaches are still at laboratory level.

- By a comparative evaluation of the advantages and disadvantages of each approach on food matrixes, safety concern and economical implication, it is suggested that the facilities of controlling temperature/humidity/gas composition and the equipment of irradiation can be used on solid food storage, photodynamic treatment, plasma treatment and electrolyzed oxidizing water can be developed for decontamination during food processing in developed countries;

while cleaning, milling and sorting before storage and eating, as well as chemical antifungal agents or biological metabolites with safe limits added in processed foods are good options for developing countries (refer to Table. 2-5). In addition, the simultaneous application of multiple approaches can achieve the maximum decontamination effects with the minimal safety concerns.

5.1.2 Fungal inhibition by mandarin peel extracts

- The inhibition of fungal growth by mandarin peel extracts is a biological control approach. Different from plant essential oils (refer to 2.2.3.2), this work focused on the antifungal activity of citrus solvent extracts.

- The present study first compared the anti-*A. flavus* activity of mandarin peel crude extracts extracted by food-grade solvents, and the ethanol crude extract showed higher antifungal activity than water crude extract.

- In general knowledge, the stability of polyphenols would be affected by high temperature (Moure et al., 2001). However, the present study demonstrated the mandarin peel polyphenols were not lost by volatilization or thermal decomposition, so that the antifungal activity of extracts could be retained after autoclaving. This result also provided evidence on potential usage of antifungal citrus extracts in heat processing.

- The moderate-polar resins were usually used in polyphenol purification (Li and Chase, 2009; Karakaya, 2011; Meng et al., 2017a). In the present study, optimized SPE with amberlite (R) XAD7HP resin was applied in separating the polyphenol-rich fraction from crude extract, resulting in the improvement of antifungal activity by isolating and concentrating polyphenols in mandarin peel extract, and removing the ingredients that promoted fungal growth (e.g. sugars).

- To identify the phenolics in mandarin peel extracts, a HPLC method was optimized according to previous study (Huang et al., 2018). The results provided evidence of polyphenols in the peel of mandarin species (*C. reticulata*).

5.1.3 AFB_1 adsorption by CCP

- The adsorption of AFB_1 by corn by-products from aqueous solution is a biological detoxification approach as well. To best our knowledge, this is the first time that the CCP was found to have fast and high AFB_1 removal ability as an adsorbent. Compared to microbial materials, CCP generally showed higher % adsorption (refer to 2.3.3). The adsorption effect of CCP was also similar with that of chemical adsorbents (refer to Table. 2-3), but CCP, as an agricultural by-product, was safer and easier-available than those chemical adsorption materials.

- The % adsorption of CCP could be affected by particle size, ratio of powder:AFB_1, temperature, and pH. However, under most of pH conditions and with particle size smaller than 500 μm, adsorption ability was not significantly impacted. The desorption study indicated the AFB_1 could be easily eluted and collected from CCP.

- For the exploration of adsorption mechanism, the results of model fitting indicates that the adsorption followed the pseudo-second-order model, and fitted the Langmuir isotherm well. In terms of the results of thermodynamic study, the adsorption of AFB_1 by CCP was a randomness reduced, exothermic and non-spontaneous process.

- The present study demonstrated the hydrophobic interaction might mainly occur between lignin (one of the major compounds in corn cob) and AFB_1, while cellulose and (arabino)xylan did not play the significant role. Furthermore, the % adsorption of the comparison with untreated corn cob and its major compounds indicated that the microstructure of CCP should be an indispensable reason in the AFB_1 adsorption.

5.2 Proposed mechanisms for *A. flavus* inhibition and AFB_1 adsorption

5.2.1 Antifungal properties of mandarin polyphenols

Polyphenols, and flavonoids in particular, have been shown to be effective antifungal agents a large range of pathogenic fungi, including *Candida* sp., *Aspergillus* sp., *Trichophyton* sp., *Pestalotiopsis* sp., *Penicillium* sp., *Fusarium* sp., etc. with different range of MICs (Al Aboody and Mickymaray, 2020). The inhibition activity might be because the properties of the polyphenols, which include the hydrophobicity/hydrophilicity, molecule size, structure (e.g. number and position of hydroxyl groups, their substitutions, with/without glycosylation and its position) (Makarewicz et al., 2021). In recent years, the mechanism of antifungal agents have also been discussed in depth (Al Aboody and Mickymaray, 2020), but limited studies focused on the mechanism of antifungal activity of citrus polyphenols. Therefore, this section mainly discusses the potential antifungal mechanism of polyphenols from mandarin peel extracts:

- Inhibition of fungal cell wall synthesis. Fungal cell wall is a characteristic structure mainly composing with glucans, chitin and glycoproteins (Garcia-Rubio et al., 2020). The antifungal mechanism could be based on the inhibition of glucans and chitin synthesis resulting in deformation of fungal cell wall (Al Aboody and Mickymaray, 2020). For instance, *trans*-cinnamaldehyde (Fig. 5-1) was investigated as a non-competitive inhibitor of glucan synthase and chitin synthesis (Chs isozymes) in fungal cell wall reducing the synthesis of *β*-(1,3)-glucan and chitin in fungi (Bang et al., 2000). In addition, as this inhibitor has the slightly different chemical structure with *p*-coumaric acid (Fig. 3-5a) and ferulic acid (Fig. 3-5d) (*p*-coumaric acid is a carboxylic acid having the -COOH group replacing the position of -CHO group in *trans*-cinnamaldehyde, with a -OH group at para position of benzene ring, and that of ferulic acid is *p*-coumaric acid with a $-OCH_3$ group at meta position of benzene ring), *p*-coumaric acid and ferulic acid also displayed prevention capability of biofilm formation of *Escherichia coli*, but had different activity compared to *trans*-cinnamaldehyde (Kot et al., 2015). Therefore, similar with *trans*-cinnamaldehyde, both *p*-coumaric acid and ferulic acid might have inhibitory activity against cell

wall synthesis.

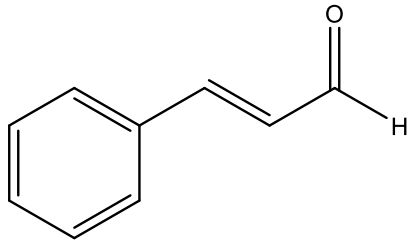

Fig. 5-1 Chemical structure of *trans*-cinnamaldehyde.

- Plasma membrane disruption leading to leakage of intracellular components. The inhibitory mechanism of polyphenol might be related to the hydrophobicity, which is correlated to the $Log_{10}P$ of polyphenols and their partition in the cytoplasmic fungal membranes. This lipophilicity causes a high affinity for fungal membrane affecting both the lipid ordering and the bilayer stability by the interaction between polyphenols and membrane, and the membrane integrity then decreases and proton passive flux increases. It is believed that this effect is mainly found with the compound with $Log_{10}P$ >3 (Ben Arfa et al., 2006). Sinensetin, nobiletin and tangeretin with $Log_{10}P$ >3 in the extracts of present study might have higher impact on fungal plasma membrane integrity.

Furthermore, the ergosterol content in fungal cell membrane and its biosynthesis could be suppressed. The ergosterol is an important component for formation of cell membranes, which normally is the target that antifungal drugs act on (Al Aboody and Mickymaray, 2020). Fisetin was found to inhibit the growth of *Cryptococcus neoformans* sp. by reducing the level of ergosterol (21.6 to 25.4%) (Reis et al., 2016), whereas caffeic acid could not only reduce the content of ergosterol of *Trichophyton rubrum*, but also repress the genetic expression (ERG1, ERG6 and ERG11 genes) for ergosterol synthesis (Cantelli et al., 2017). Although there is less evidence show the effects of citrus polyphenols on ergosterol content and biosynthesis, the polyphenols in present mandarin extracts might have similar function due to the close carbon skeleton structure of fisetin (Fig. 5-2a) with sinensetin (Fig. 3-5j), nobiletin (Fig. 3-5k) and tangeretin (Fig. 3-5l), and caffeic acid (Fig. 5-2b) with *p*-coumaric acid (Fig. 3-5a) and ferulic acid (Fig. 3-5d).

(a) (b)

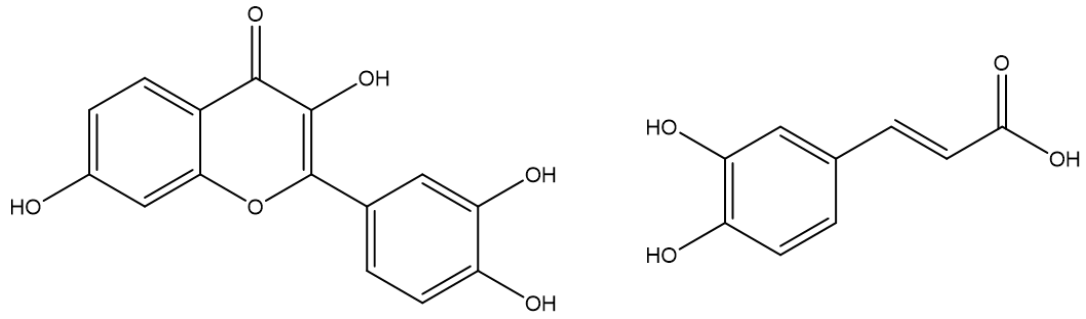

Fig. 5-2 Chemical structure of fisetin (a) and caffeic acid (b).

- Suppression of fungal DNA and DNA enzyme function. In previous studies, flavonoids of B ring have been demonstrated to intercalate or form hydrogen bond of nucleic acid bases in bacteria (Makarewicz et al., 2021); similarly, *p*-coumaric acid could also locate in the hydrophobic interior of DNA or intercalate into the bases, as well as the complex was could be stable by stacking interaction with DNA bases (Lou et al., 2012). Thus, polyphenols could bind with nucleic acid to inhibit the cellular functions after the compounds diffusing through the cell membrane and penetrating into microbial cells. This suggests that some of extracted polyphenols from mandarin peel might be able to inhibit fungi by interfering the DNA functions.

DNA topoisomerases are crucial enzymes joining DNA replication, transcription, recombination, chromosome condensation and segregation, and DNA repair, that can be found in many prokaryotic and eukaryotic organisms (Kim et al., 2002). The mechanisms of flavonoids against topoisomerases were predicted as a): -OH group at position of C4’ in B ring is the key for the flavonoid to inactive the enzyme, additionally -OH group at position of C3’ and C5’ that improves the redox activity of the B ring and makes the compound as a redox-dependent poison; and b) 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 reduction of the ability to function (Makarewicz et al., 2021). As eriocitrin (Fig. 3-5b), rutin (Fig. 3-5c), taxifolin (Fig. 3-5e), narirutin (Fig. 3-5f) and eriodictyol (Fig. 3-5i) all have the elements mentioned above, mandarin peel extracted flavonoids might be able to affect bioactivity of DNA metabolite enzymes.

- Reduction of energy metabolism. ATP is produced by the oxidative phosphorylation or photophosphorylation of ATP synthase bound with cell membrane and mitochondria membrane (Chinnam et al., 2010). Chinnam et al. (2010) tested the inhibition of 17 flavonoids to ATP synthase activity in *Escherichia coli*. Among the flavonoids, hesperidin, diosmin and rutin showed about 40 to 60% inhibition of enzyme activity. This might suggest that these compounds could mitigate the ATP synthase activity of *Aspergillus* sp. to some extent as well.

- In addition, Ponts et al. (2011) found ferulic, coumaric, caffeic and syringic acids caused toxin accumulation in *F. graminearum*, and the higher antioxidant properties of the phenolic acids, the higher effect of the compounds on fungal growth. Al Aboody and Mickymaray (2020) reviewed the efflux pumps, transport proteins for moving toxic substances out from fungal cells, could be inhibited by many polyphenols (as well as treated with antifungal drugs sometimes), such as apigenin, baicalein, curcumin, apigenin, chrysin, luteolin, tangeritin. However, there are very limited studies discussed above promising antifungal mechanisms of most common phenolic compositions in citrus fruits related to *Aspergillus* sp. growth. These promising actions could be investigated in further research.

5.2.2 AFB_1 adsorption by CCP

Studies of solute adsorption from liquid system have been investigated for decades. Various materials are employed as adsorbents including alumina, clays, zeolites, silica gel, activated carbon/biochar, microbial biomasses, polymeric matters and novel composite materials (Sabzehmeidani et al., 2021; Chai et al., 2021). According to different chemical properties of adsorbents or adsorbates, the adsorption normally follows with multiple mechanisms. For inorganic compounds, the adsorption of adsorbates onto adsorbent is following the mechanisms of electrostatic interaction, ion exchange/cation exchange, precipitation and complexation; while, for organic compounds, the mechanisms are mainly partitioning, pore filling, electrostatic interaction, electron donor and acceptor interaction and hydrophobic interaction (Abbas et al., 2018). It is worth noting that the adsorption is complicated that could be controlled by multi-

mechanisms rather than any single mechanism. Thus, this section mainly discusses the potential adsorption mechanism of AFB_1 on CCP:

- Interaction between CCP and AFB_1 in adsorption. AFB_1 (Fig. 4-3f) is a non-ionizable molecule and does not have hydrophilic groups, such as -COOH and -OH (Ma et al., 2021). As the surface of natural cob is not charged, the adsorption of AFB_1 on CCP may not be electrostatic interaction, and that might be the reason why the adsorption was less affected by pH (Abbas et al., 2018). Similarly, electron donor and acceptor interaction mainly occurred in aromatic compounds adsorbed on the adsorbents with graphene-like surface, which may not be the case for the adsorption of the heterocyclic compound, AFB_1, on natural CCP surface (Abbas et al., 2018).

On the contrary, the hydrophobic interaction might be the mechanism behind the adsorption of AFB_1 on CCP surface. Hydrophobic interaction happened between hydrophobic surface of adsorbents and hydrophobic organic compound (Abbas et al., 2018). Although corn cob gave strong signals in some hydrophilic characteristic peaks (e.g. -OH stretching of hydroxyl groups, -CO groups stretching in ether), there were also hydrophobic groups (e.g. -CH, -C=O which was found in lignin as well (Derkacheva and Sukhov, 2008)) might have hydrophobic interaction with AFB_1 (Ojedokun and Bello, 2017). In a study of AFB_1 removal by metal-organic framework materials, with the carbonization temperature arise (uncarbonized, 400°C, 600°C and 800°C), the intensities of -OH peaks in the material gradually missed, suggesting the increasing of hydrophobic of the material surface. The sample carbonized at high temperature (600°C and 800°C) displayed the highest AFB_1 removal capability (Ma et al., 2021). Thus, the hydrophobic treatment of corn cob may improve the AFB_1 adsorption capability to some extent.

In spite of the -OH groups in the natural CCP could not form hydrophobic interaction with AFB_1 molecule, they can also establish hydrogen bonds with 'O atoms' of -C=O groups of AFB_1, which might provide strong dipole-dipole interaction in the adsorption (Rasheed et al., 2020). An acid modified could slightly induce -C=O groups to corn cob that may cause further increasing of AFB_1 adsorption (Hu et al., 2018).

- Role of complicated microstructure and morphology of CCP surface. It was demonstrated that the adsorption performance of adsorbates could be greatly affected by specific surface area

(S_{BET}), pore volume (V_P) and pore size of adsorbents (Ma et al., 2021).

In general, larger surface area and pore volume causes a higher adsorption capacity on adsorbents, as it can provide more sorption sites and space. For instance, Wang et al. (2018) reported the equilibrium adsorption capacity of AFB_1 by montmorillonite with 0.5 CEC of octylphenol polyoxyethylene ether (0.5OP-10Mt, 36.967 m^2/g for S_{BET}, 0.087 cm^3/g for V_P) was around 0.5 mg/g higher than that by montmorillonite with 1.0 CEC of octylphenol polyoxyethylene ether (1.0OP-10Mt, 15.920 m^2/g for S_{BET}, 0.035 cm^3/g for V_P). Ying et al. (2021) tested the DON adsorption effect of carbonized soybean dregs (SDB-6-X) synthesized with KOH (3655.95 m^2/g for S_{BET}, 1.936 cm^3/g for V_P), K_2CO_3 (1444.46 m^2/g for S_{BET}, 0.651 cm^3/g for V_P) or $KHCO_3$ (1443.67 m^2/g for S_{BET}, 0.635 cm^3/g for V_P), and SDB-6-KOH was found having the best performance. However, there were also some research findings that were not in in accordance with this rule due to the specificity of adsorbents' adsorption behavior. For example, although the S_{BET} and V_P of amorphous calcium silicate hydrate (ACSH) was around 30 m^2/g and 0.2 cm^3/g larger than that of crystalline calcium silicate hydrate (CCSH) respectively, the adsorption capability of AFB_1 of CCSH was 3.4 times higher than ACSH. This is because the adsorption of AFB_1 by ACSH was surface adsorption, while that by CCSH was interlayer adsorption, which was higher than the surface adsorption (Zeng et al., 2014). In the description above, when the sorption sites on 0.5/1.0OP-10Mt were sufficient for AFB_1 adsorption, the larger S_{BET} and V_P, the higher adsorption capacity. Whereas, 2.0OP-10Mt had small S_{BET} (6.460 m^2/g) and V_P (0.017 cm^3/g) that did not provide enough sorption sites, and the adsorption ability of 2.0OP-10Mt was lower than that of 0.5OP-10Mt but higher than 1.0OP-10Mt. This is because the existence of ion-dipole interaction and coordination between cations of these adsorbents and carbonyl groups on AFB_1 molecule was also played an important role in the adsorption (Wang et al., 2018). As for raw corn cob, the S_{BET} and V_P was 93.46 m^2/g and 0.0577 cm^3/g respectively (Chukwuemeka-Okorie et al., 2018), so that the CCP could provide large amount sorption sites and space for AFB_1 adsorption. When the particle size was larger than 500 μm, it would be difficult for AFB_1 molecule diffuse to the center of 'big' CCP mass, so this would limit the adsorption capacity of AFB_1 by CCP.

In addition, the adsorption also depends on molecular size of adsorbates and pore size of adsorbents. When the molecule size of adsorbates is larger than pore diameter, there is no

adsorption process occurring because of the steric hindrance; when the molecule size approximately equal to pore diameter, the adsorbates can be strongly captured by the adsorbents and no easy desorption due to the superposition of potential energy fields in adjacent wall pores; while the molecule size of adsorbates smaller than pore diameter can take place capillary condensation easily in the pores of adsorbates to improve the adsorption capacity (Zhu et al., 2020). For AFB_1 molecule, its surface area is around 1.38 nm^2 (Ma et al., 2021). Besides, the micropores (<2.0 nm) of the adsorbents were found to impede the diffusion of AFB_1, while the AFB_1 could be directly adsorbed with mesopores (2.0 to 50.0 nm) by the capillary effect (Galvano et al., 1996; Ma et al., 2021). This could be found in the adsorption by activated charcoal (Galvano et al., 1996), magnetic silica prepared from rice husk (Li et al., 2020) and modified montmorillonite (Wang et al., 2018). The average pore sizes of CCP were presented to be about 18 to 25 nm, and mesopores took up 27.40% of total pores in the raw cob (Ji et al., 2015; Chukwuemeka-Okorie et al., 2018; Olatunji et al., 2020). Thus, it could be believed that the adsorption of AFB_1 by CCP might be the capillary effect happened in the mesopores of corn cob.

5.3 Limitations of this research and future work

- Extraction efficiency performs variably under different extraction conditions including time, water/organic solvent ratio, temperature and method (e.g. soaking and shaking, ultrasonication) (Lapornik et al., 2005; Altıok et al., 2008; Louis et al., 2011; Gómez-Maldonado et al., 2020). However, this work only focused on the extraction under one condition. However, in order to obtain the maximum extraction efficiency, various extraction conditions, including temperature, extraction solvent and extraction methods, should be optimized in the further studies.

- The concentrations of crude extract that could completely inhibit *A. flavus* tested in the model system were too high to be applied in foods. Thus, in practical fungal mitigation, the optimum inhibitory concentration applied in real food systems should be understanded. In addition, the utilization of extract can focus on SPE fraction in the future.

- In general, the plant metabolites having antifungal activity could make the changes on fungal morphology, cellular metabolism, mycelia growth, spore germination or mycotoxin production (de Alvino Leite et al., 2014; Yang and Jiang, 2015; Sun et al., 2016; Hu et al., 2021). For citrus extracts, it is also interesting to understand how the extracts impact the fungal development and metabolism.

- In the food industry, the contamination of various natural fungus strains occurs simultaneously depending on the conditions of food storage and production chain (Greco et al., 2019). Therefore, more toxigenic fungus strains should be investigated in the antifungal study of mandarin extracts, *Penicillium* sp. and *Fusarium* sp. in particular.

- The adsorption capability of same adsorbents to different toxins may not always have the same performance (Avantaggiato et al., 2013; Greco et al., 2019). Hence, corn cob as the adsorbent should be tested for the adsorption of more mycotoxins (e.g. FBs, DON, ZEN, etc.) in the future studies. In some cases, a variety of mycotoxins produced by different fungi may coexist in one system. In spite of there was no competitive adsorption of multi-mycotoxin on gape pomace (Avantaggiato et al., 2013), it is interesting to know if different mycotoxins would compete for adsorption on CCP.

- Generally, chemical modification (e.g. strong acid, strong alkali) and carbonization could result in a change of adsorbent microstructure or surface properties so that increased their adsorption capability (Palanivell et al., 2019; Muscarella et al., 2021; Cheng et al., 2021). Similarly, these methods can be used for CCP modifications, to test if the adsorption capability of AFB_1 would be improved.

5.4 Application of citrus extracts and corn by-products towards inhibition of fungal and AFB_1 removal

5.4.1 Recommendations for practical application of agricultural by-products in the mitigation of *A. flavus* and AFB_1

In the practical application (Table. 5-1), the mandarin extracts, SPE fractions in particular, can be added in antifungal spray as a replacement of chemical fungicide. However, this approach would bring extra moisture and remain the antifungal ingredients to the foods (e. g. cereals). The extracts can be applied as food preservatives in processed foods (e. g. breads, biscuits), but the addition of high concentration of extracts may alter the flavour of foods.

The CCP would be mainly used to remove AFB_1 from liquid foods, including cereal milks, oils, grain alcohols, while CCP could perform differently in various food matrixes (Barrientos-Velázquez et al., 2016), and the adsorbent may cause nutrient losses due to the adsorption of nutrient ingredients by adsorbent (Ma et al., 2021). Additionally, corn by-products are being used in animal feeding (Rostika and Safitri, 2012; Wachirapakorn et al., 2016), thus it is interesting to investigate the reduction of AFB_1 exposure by these materials under gastrointestinal environment, and the impact of adsorbent on the adsorption of nutrients by the body. The CCP can be also applied as the padding of column or a material of manufacturing filter for removal of AFB_1 from contaminated water, so this powder could be used while washing the cereal before eating. When the storage and production is raised to up-scale, the cost and environmental impact caused by time and fuel consumption during extraction and drying process will also be increased (Table. 5-1). Besides, before the production of antifungal extracts, citrus by-products undergo cleaning peeling, dehydration, milling, sieving, and storage. On the one hand, the drying steps increase the porosity of the waste matrix, which accelerate the diffusion rate of solvent and improve the extraction efficiency; on the other hand, these steps can decrease extraction yield of polyphenols by high temperature, oxygen, light or a high residence time (M'hiri et al., 2014). Similarly, milling causes to the breakdown of citrus cell structures leading to the enhancement of extraction yield of the antifungal compounds (M'hiri et al., 2014), while the large surface area of small particle

size also results in the accelerated loss of sensitive bioactive compounds. Therefore, the conditions of pre-treatment can be investigated before solvent extraction step to achieve the maximum extraction yield of citrus materials. During the extraction and purification steps, the extraction solvents and SPE resin can be recycled and reused (Table A-7-9) for conservation of resources. In order to maximize the utilization of agricultural by-products and reduce the energy loss in production processing, the combination of a variety of processing steps of value-added products is a way worth to be performed in industrial production as well. So, as a pectin-rich material, the residual of citrus peel by-product after polyphenol extraction can continuously to be used in the production of pectin (Ayora-Talavera et al., 2017).

For AFB_1 removal, the current research on agricultural by-products as mycotoxin adsorbents mainly focus on the agricultural product-based biochar and novel synthesis materials (Zahoor and Ali Khan, 2014; Ying et al., 2021; Li et al., 2020). As the CCP is easy to suspend in the liquid, it would be difficult to separate from system during use. Thus, the CCP is also able to link with magnetic particles (e.g. iron nitrate, Fe_3O_4 particles), and this magnetic cob powder therefore can be easily collected/removed with magnetic field after adsorption process (Li et al., 2020; Bai et al., 2021). Although the modified agricultural materials can be inexpensive, have stable chemical properties and show good adsorption capability even higher than raw materials (Meng et al., 2017b; Rojas et al., 2019), it also needs to be considered that the usage of chemicals and the energy consumption during processing increasing the costs and causing huge environmental burden. Similarly, the extraction of polysaccharides from plant materials can be added previous to the CCP being used as adsorbent (Hu et al., 2018).

5.4.2 Safety concerns in application

Although citrus polyphenols, flavonoids in particular, have been found to show many health benefits on human, excessive intake could also be potentially toxic to health, such as mutagenicity, genotoxicity, cytotoxicity and effect on thyroid hormone production (Skibola and Smith, 2000). Yet, studies on acceptable daily intake of specific flavonoid are still limited to date. In a review published by Del Bo et al. (2019), the established daily intake of total polyphenol was found to be

up to 1.5 mg/day worldwide, and total flavonoid intake was up to 0.6 mg/day, which varied in terms of geographical area, dietary habits, age, gender, education level and socio-cultural factors. Thus, dietary exposures under these levels are not likely to cause adverse health effects. Based on the dose of highest polyphenol intake, the safe doses in use should be lower than the calculated values of 20.78 g for water elution fraction or 18.15 g for ethanol elution fraction of mandarin extracts.

Corn cob is a porous, lightweight, non-allergic, non-toxic, biodegradable and odourless material (Ji et al., 2015). Thus, the natural CCP itself would not bring toxicity to human as adsorbent. Whereas it is worth to noting the new safety risk caused by the mildew of natural CCP in use for long time.

5.4.3 Current status of mycotoxin regulation and recommendations for policy making

In order to reduce the effects of mycotoxins on human and animal health, around 100 countries and regions have formulated the mycotoxin regulations since the 21st century, which covers 87% of the global population (FAO, 2003). The regulations mainly stipulate the content limitation of mycotoxin in foods and feeds, to minimize the availability of contaminated foods by human. Nevertheless, some studies reported that public consciousness and education is another important factor in whether people would be exposed to mycotoxin contamination. The lack of awareness and education of health hazards associated with mycotoxins usually causes more direct or indirect exposure to the contamination (Misihairabgwi et al., 2019). Therefore, while continuing to scientifically develop the regulations relating to mycotoxins in foods, the education of awareness to the public should be strengthened (Table. 5-1). Meanwhile, the education can also involve teaching people the correct storage and cleaning methods, and encouraging people to use safe antifungal and mycotoxin reduction products in daily life, e.g. using ‘CCP cleaning bag’ while washing cereals.

For large-scale storage and production, strict monitoring and testing of fungal and mycotoxin

contamination should be performed during storage, before and after processing. In addition, cleaning step should be mandatorily added in food production to remove potential contaminants involved in processing (Table. 5-1).

Table. 5-1 Recommendations, applications, concerns and limitations of decontamination materials in this research at different levels

	Research in Model Systems	Research in Food Systems	Large Scale Adaption	Policy and Regulations
Antifungal Agent	• Performed in solid or broth culture medium to inhibit fungal growth, spore germination or aflatoxin production	• Used as an ingredient or replacement of fungicide spray for cereal storage • Added into cereal-based products as an antifungal additive • May mitigate a variety of natural fungi co-occurring on foods	• Fuel and time cost are the major costs in solvent extraction and SPE isolation (drying step) • Reduce environmental burden by agricultural waste, but may cause environmental pollution from energy consumption during extraction and drying process • May have safety issue due to the high MIC	• For consumers and small-scale farmers, improve them with the awareness of health hazards by mycotoxins, and educate them the proper handling and processing approaches for contaminated foods • For large-scale farms, strength the monitor and management of contamination during pre-, post-harvest, and storage • For food industry, mandatorily add 'clean procedure' to reduce the involvement of contaminants, and test the food products before market
Adsorbent	• Remove aflatoxin from aqueous solutions under certain conditions	• Mainly used in liquid food matrixes, e.g. cereal milk, oils, grain alcohol • Added into animal feeds or taken as supplements to reduce aflatoxin exposure • Used as AFs-contaminated water cleaner • May apply in the food systems accumulating multi-mycotoxins	• Energy cost is the major cost in the preparation of adsorbent (drying and milling step) • Reduce environmental burden by agricultural waste, but may cause environmental pollution from energy consumption • May be at risk of mildew because of long-time soaking in water	
Scale	100 g	100 kg	10 tonnes	-

5.5 Conclusion

Since the mycotoxins were discovered, human have never stopped eliminating the loss and health risk caused by the toxins and their producer. Many techniques, classified as physical, chemical and biological approaches, have been investigated for mitigating fungal growth/mycotoxin production and mycotoxin detoxification respectively. The decontamination approaches are applicable to different foods depending on their matrixes. But in any case, the efficient, low-toxicity, cost-effective and easy-operated approaches have always been the primary purpose to researchers.

Also to achieve this aim, agricultural by-products were tested for decontamination activity of both

fungi and mycotoxin. In the antifungal study, the work was mainly focusing on investigating the mandarin peel extracts on growth inhibition of *A. flavus*, and developing a SPE method for isolating the antifungal effective fraction from the extracts and improving the antifungal activity. In the mycotoxin reduction study, the effect of different factors on the AFB_1 adsorption by CCP was explored, and meanwhile, the physicochemical properties of adsorption were demonstrated by theoretical studies.

The findings in this study provide evidence that mandarin peel extracts had the antifungal ability, especially the mandarin polyphenol-rich fractions isolated by SPE showed a higher inhibitory efficiency than crude extracts. After the identification of polyphenols, 10 of flavonoids and 2 of phenolic acids were found in the extracts. Besides, corn con showed a high aflatoxin adsorption capability, and the adsorption was not significantly influenced by particle sizes, temperature and pH. The adsorption was a monolayer, randomness reduced, exothermic and non-spontaneous process. This result revealed the huge potential on decontamination of fungi and mycotoxins. Additionally, the effectiveness, feasibility and cost-effectiveness of using agricultural by-product-based materials for decontamination in practical application needs to be further studied.

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Appendix: Supplementary Data for Chapter 3

A.1 Material and methods

A.1.1 Effect of autoclaving on antifungal activity of crude mandarin extracts

To avoid microbial contamination during *A. flavus* culture, mandarin extracts were autoclaved prior to their use in the antifungal assay at 121°C for 15 min. Citrus crude extracts (150 mg) were added to 15 mL of molten potato dextran agar (PDA) and stirred for one minute. Fungi were inoculated on the agar. The dishes were incubated at 30°C under dark conditions for 7 days. The diameter of fungal colony was measured with a ruler to 1 mm every day.

A.1.2 Determination of the effect of crude mandarin extracts on mycelia growth and AFB_1 production of aflatoxigenic strain

In mycelia growth study, the aflatoxigenic strain (*A. flavus* NNRL 3375, Cranfield University) was cultured on yeast extract sucrose agar (YES agar: 4 g of yeast extract, 20 g of sucrose, 1 g of KH_2PO_4, 0.5 g of $MgSO_4$ and 15 g of agar dissolved in 1 L of distilled water) at 30°C under dark conditions. The procedure of determining mycelia growth inhibited by mandarin extracts (10 mg/mL) was as described in 3.2.5.

For the determination of AFB_1 produced by aflatoxigenic *A. flavus*, the strain was cultured in YES broth at 30°C in a shaking incubator (150 rpm) (Progen Scientific, UK). Spore suspension (200 µL) obtained from 2-day-old culture broth was added to 5 mL of autoclaved YES broth containing mandarin peel extracts at concentration of 10 mg/mL, and incubated for 4 days until well grew. The control group was the culture broth without extracts. After incubation, the culture broth was centrifuged at 4000 rpm for 15 min. Then, 1 mL of supernatant was collected and loaded to pre-conditioned Oasis Max column (Waters, UK) to isolate AFB_1 from broth. Later, the column was washed with 5 mL of distilled water, and then AFB_1 was eluted with 2 mL of methanol. Finally,

the AFB_1 elution solution was diluted to 4 mL with water. The concentration of AFB_1 was detected by HPLC as described in 4.2.7.

A.1.3 Optimization of SPE procedure

The optimization of SPE procedure investigated the effect of resin doses and repeat SPE on separation effect of sugars, TPC and TFC of mandarin peel extracts. Before SPE, 5 mL of original water or ethanol extract solution was dried by Genevac and then re-dissolved in 1 mL of water. The resin was activated with 5 mL of water, ethanol and water in sequence. Next, the activated resin was added into the 1 mL of pre-concentrated sample, and stand for 24 h at room temperature. Then, the resin was separated from extract solution, and the rest of solution was kept as filtrate solution. The resin was washed with 2.5 mL of distilled water for three times (1^{st} wash, 2^{nd} wash, 3^{rd} wash), and finally eluted with 2.5 mL of ethanol for three times (1^{st} elution, 2^{nd} elution, 3^{rd} elution). For better SPE results, the filtrate solution and three wash solutions after first SPE were collected and done for second SPE. In order to reduce extraction costs, the amberlite (R) XAD7HP resin was tested for reuse.

The determination of TPC and TFC was as described in 3.2.7.1 and 3.2.7.2. The determination of sugar contents was as described below.

A.1.4 High performance anion exchange chromatography with pulsed amperometric detector (HPAEC-PAD) analysis of sugar contents in mandarin peel extracts

The sugars were determined by HPAEC-PAD method as previous reported by (Scharf et al., 2018). The analysis was carried out with Dionex ICS-5000 ion chromatography system (Thermo Scientific) comprising a carboPac Pa20 analytical column (3 x 150 mm with a capacity of 65 µeq), maintained at 20°C. The mobile phase consisted of solvent A (Milli-Q water) and solvent B (300 mM NaOH) with the flow rate of 0.4 mL/min. The gradient elution was performed as follows: 0-10 min, 30% B; 10-15 min, 30-100% B; 15-18 min, 100% B; 18-20 min, 100-30% B; 20-25 min, 30% B. The injection volume was 10 µL. The final result of carbohydrate detection as deduced from

the area (nC) of the chromatogram was calculated as percentage soluble sugars in dry weight (μg).

A.2 Results

A.2.1 Effect of autoclaving on antifungal activity of crude mandarin extracts

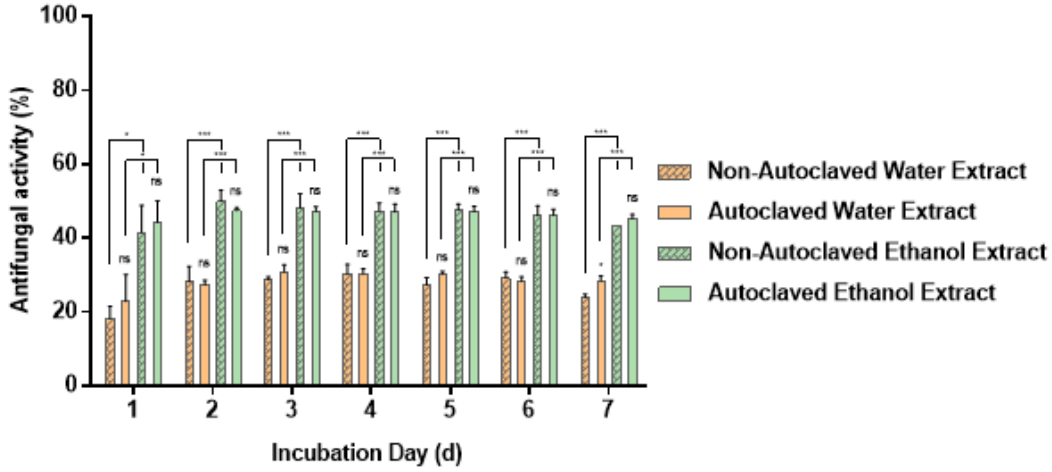

Fig. A-1 Antifungal activity (%) of mandarin peel extracts (10 mg/mL) at 30°C in 7 days. * represents significant differences between non-autoclaved samples and autoclaved samples. ***, $p \leqslant 0.0001$; **, $p \leqslant 0.001$; *, $p \leqslant 0.05$ and ns, $p > 0.05$. Results are means of three replicated dishes.

A.2.2 Effect of crude mandarin extracts on mycelia growth and AFB_1 production of aflatoxigenic strain

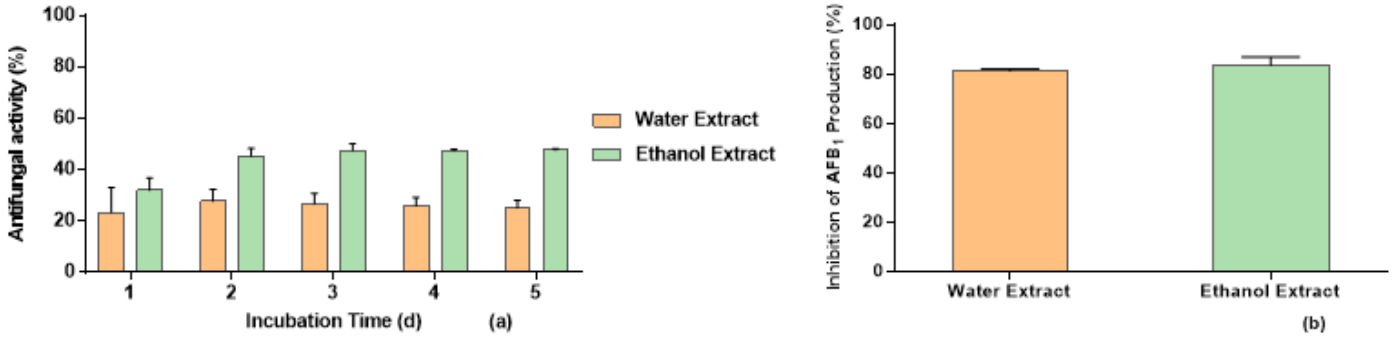

Fig. A-2 Antifungal activity (%) of mandarin water and ethanol extracts (10 mg/mL) at 30°C for 5 days (a), and Inhibition (%) of AFB_1 production with mandarin water and ethanol extracts (10 mg/mL) at 30°C on Day 4 (b).

A.2.3 Optimization of SPE procedure

A.2.3.1 Separation effect with different doses of resin

Table. A-1 The sugar contents in mandarin peel extracts

Extraction	Resin Dose	Sugars	Contents in 5 mL Original Extract Solution (μg)													
			Original Solution	Filtrate		1st wash		2nd wash		3rd wash		1st elution		2nd elution		3rd elution	
					Recovery (%)[a]		Recovery (%)		Recovery (%)		Recovery (%)		Recovery (%)		Recovery (%)		Recovery (%)
Water	400 mg	Glucose	9812.03	7620.14	77.66	2272.50	23.16	345.14	3.52	53.79	0.55	127.29	1.30	31.65	0.32	-	-
		Fructose	14127.23	8914.29	63.10	2695.46	19.08	401.70	2.84	74.75	0.53	148.61	1.05	5.64	0.04	-	-
		Sucrose	5586.45	4533.42	81.15	1723.73	30.86	196.75	3.52	45.33	0.81	19.07	0.34	7.88	0.14	-	-
	600 mg	Glucose	9812.03	6884.03	70.16	2997.61	30.55	424.30	4.32	89.67	0.91	135.34	1.38	33.28	0.34	-	-
		Fructose	14127.23	8198.14	58.03	3511.83	24.86	561.86	3.98	99.73	0.71	210.57	1.49	5.85	0.04	-	-
		Sucrose	5586.45	4210.51	75.37	2220.69	39.75	46.04	0.82	74.54	1.33	25.74	0.46	6.19	0.11	-	-
	800 mg	Glucose	9812.03	7091.46	72.27	4208.48	42.89	467.59	4.77	0.87	0.01	96.11	0.98	44.71	0.46	-	-
		Fructose	14127.23	8300.10	58.75	4882.58	34.56	643.62	4.56	90.34	0.64	132.07	0.93	5.00	0.04	-	-
		Sucrose	5586.45	4317.89	77.29	3099.57	55.48	238.48	4.27	83.95	1.50	19.92	0.36	10.62	0.19	-	-
Ethanol	400 mg	Glucose	7614.82	6420.02	84.31	1750.17	22.98	347.96	4.57	29.43	0.39	78.99	1.04	30.45	0.40	-	-
		Fructose	11093.04	8076.17	72.80	2175.15	19.61	459.70	4.14	33.48	0.30	105.24	0.95	6.98	0.06	-	-
		Sucrose	4319.61	3371.76	78.06	1152.19	26.67	180.22	4.17	26.41	0.61	21.96	0.51	7.10	0.16	-	-
	600 mg	Glucose	7614.82	6318.05	82.97	2472.09	32.46	353.50	4.64	74.32	0.98	100.91	1.33	37.07	0.49	-	-
		Fructose	11093.04	7604.30	68.55	3076.21	27.73	490.62	4.42	91.98	0.83	137.74	1.24	9.40	0.08	-	-
		Sucrose	4319.61	3450.10	79.87	1615.74	37.40	172.03	3.98	50.75	1.17	31.81	0.74	8.36	0.19	-	-
	800 mg	Glucose	7614.82	5931.90	77.90	2824.17	37.09	415.13	5.45	114.52	1.50	76.01	1.00	22.31	0.29	-	-
		Fructose	11093.04	7159.47	64.54	3505.86	31.60	596.09	5.37	141.50	1.28	88.77	0.80	3.89	0.04	-	-
		Sucrose	4319.61	3360.56	77.80	1868.67	43.26	203.52	4.71	78.67	1.82	19.06	0.44	3.49	0.08	-	-

[a] Recovery (%), the percentage of each fraction recovered from original extract solutions

Table. A-2 The total phenolic content in mandarin peel extracts

Extraction	Resin Dose	Original Solution	Contents in 5 mL Original Extract Solution (μg GAE[a])													
			Filtrate		1st wash		2nd wash		3rd wash		1st elution		2nd elution		3rd elution	
				Recovery (%)[b]		Recovery (%)		Recovery (%)		Recovery (%)		Recovery (%)		Recovery (%)		Recovery (%)
Water	400 mg	1905.80	515.74	27.06	226.96	11.91	95.76	5.02	8.08	0.42	594.86	31.21	268.91	14.11	70.36	3.69
	600 mg		468.87	24.60	272.57	14.30	93.95	4.93	89.89	4.72	619.57	32.51	272.21	14.28	78.62	4.13
	800 mg		513.72	26.96	384.17	20.16	157.17	8.25	101.92	5.35	563.08	29.55	287.21	15.07	85.43	4.48
Ethanol	400 mg	1453.99	381.07	26.21	112.75	7.75	55.69	3.83	0	0	356.78	24.54	218.30	15.01	76.41	5.26
	600 mg		355.39	24.44	187.46	12.89	48.80	3.36	63.01	4.33	341.49	23.49	260.11	17.89	74.09	5.10
	800 mg		351.35	24.16	204.82	14.09	74.13	5.10	75.62	5.20	346.45	23.83	215.25	14.80	65.76	4.52

[a] GAE, gallic acid equivalent

[b] Recovery (%), the percentage of each fraction recovered from original extract solutions

Table. A-3 The total flavonoid content in mandarin peel extracts

Extraction	Resin Dose	Original Solution	Contents in 5 mL Original Extract Solution (μg RE[a])													
			Filtrate		1st wash		2nd wash		3rd wash		1st elution		2nd elution		3rd elution	
				Recovery (%)[b]		Recovery (%)		Recovery (%)		Recovery (%)		Recovery (%)		Recovery (%)		Recovery (%)
Water	400 mg	1314.02	197.59	15.04	8.42	0.64	21.67	1.65	46.59	3.55	791.58	60.24	321.49	24.47	0	0
	600 mg		202.88	15.44	15.09	1.15	37.16	2.83	0	0	764.60	58.19	232.80	17.72	56.21	4.28
	800 mg		236.08	17.97	33.16	2.52	28.53	2.17	1.85	0.14	635.86	48.39	378.30	28.79	52.63	4.01
Ethanol	400 mg	1082.07	27.55	2.55	45.20	4.18	44.35	4.10	44.74	4.13	577.21	53.34	411.34	38.01	7.03	0.65
	600 mg		13.07	1.21	46.59	4.31	49.91	4.61	0	0	646.70	59.76	411.12	37.99	66.34	6.13
	800 mg		10.90	1.01	37.89	3.50	51.98	4.80	0	0	537.72	49.69	305.55	28.24	45.83	4.24

[a] RE, rutin equivalent

[b] Recovery (%), the percentage of each fraction recovered from original extract solutions

A.2.3.2 Separation effect after two repeated SPE

Table. A-4 The sugar contents in mandarin peel extracts

				Contents in 5 mL Original Extract Solution (μg)													
				Original Solution	Filtrate		1st wash		2nd wash		3rd wash		1st elution		2nd elution		3rd elution	
	Extraction	Resin Dose	Sugars			Recovery (%)[a)]		Recovery (%)		Recovery (%)		Recovery (%)		Recovery (%)		Recovery (%)		Recovery (%)
1st SPE	Water	400 mg	Glucose	9812.03	7620.14	77.66	2272.50	23.16	345.14	3.52	53.79	0.55	127.29	1.30	31.65	0.32	-	-
			Fructose	14127.23	8914.29	63.10	2695.46	19.08	401.70	2.84	74.75	0.53	148.61	1.05	5.64	0.04	-	-
			Sucrose	5586.45	4533.42	81.15	1723.73	30.86	196.75	3.52	45.33	0.81	19.07	0.34	7.88	0.14	-	-
		800 mg	Glucose	9812.03	7091.46	72.27	4208.48	42.89	467.59	4.77	0.87	0.01	96.11	0.98	44.71	0.46	-	-
			Fructose	14127.23	8300.10	58.75	4882.58	34.56	643.62	4.56	90.34	0.64	132.07	0.93	5.00	0.04	-	-
			Sucrose	5586.45	4317.89	77.29	3099.57	55.48	238.48	4.27	83.95	1.50	19.92	0.36	10.62	0.19	-	-
	Ethanol	400 mg	Glucose	9812.03	6420.02	84.31	1750.17	22.98	347.96	4.57	29.43	0.39	78.99	1.04	30.45	0.40	-	-
			Fructose	14127.23	8076.17	72.80	2175.15	19.61	459.70	4.14	33.48	0.30	105.24	0.95	6.98	0.06	-	-
			Sucrose	5586.45	3371.76	78.06	1152.19	26.67	180.22	4.17	26.41	0.61	21.96	0.51	7.10	0.16	-	-
		800 mg	Glucose	7614.82	5931.90	77.90	2824.17	37.09	415.13	5.45	114.52	1.50	76.01	1.00	22.31	0.29	-	-
			Fructose	11093.04	7159.47	64.54	3505.86	31.60	596.09	5.37	141.50	1.28	88.77	0.80	3.89	0.04	-	-
			Sucrose	4319.61	3360.56	77.80	1868.67	43.26	203.52	4.71	78.67	1.82	19.06	0.44	3.49	0.08	-	-
2nd SPE	Water	400 mg	Glucose	-	2976.76	60.68	320.77	6.54	167.52	3.41	-	-	41.28	0.84	23.84	0.49	-	-
			Fructose	-	3237.61	45.84	424.16	6.00	189.17	2.68	-	-	44.15	0.63	24.29	0.34	-	-
			Sucrose	-	1758.98	62.97	0	0	84.65	3.03	-	-	27.34	0.98	10.47	0.37	-	-
		800 mg	Glucose	-	3255.32	66.35	351.04	7.16	176.14	3.59	-	-	33.81	0.69	11.37	0.23	-	-
			Fructose	-	3783.68	53.57	443.56	6.28	208.62	2.95	-	-	36.72	0.52	12.25	0.17	-	-
			Sucrose	-	1996.74	71.49	0	0	91.42	3.27	-	-	23.63	0.85	6.89	0.25	-	-
	Ethanol	400 mg	Glucose	-	2557.20	67.16	386.81	10.16	140.19	3.68	-	-	24.95	0.66	2.33	0.06	-	-
			Fructose	-	3132.08	56.47	380.40	6.86	169.33	3.05	-	-	26.43	0.48	2.03	0.04	-	-
			Sucrose	-	1363.73	63.14	0	0	60.26	2.79	-	-	12.47	0.58	1.09	0.05	-	-
		800 mg	Glucose	-	2674.13	70.23	333.32	8.75	143.24	3.76	-	-	30.09	0.79	7.02	0.18	-	-
			Fructose	-	3392.60	61.17	334.67	6.03	158.56	2.86	-	-	33.24	0.60	5.76	0.10	-	-
			Sucrose	-	1509.34	69.88	0	0	73.55	3.41	-	-	15.05	0.70	2.95	0.14	-	-

[a)] Recovery (%), the percentage of each fraction recovered from original extract solutions

Table. A-5 The total phenolic content in mandarin peel extracts

	Extraction	Resin Dose	Contents in 5 mL Original Extract Solution (μg GAE[a])													
			Original Solution	Filtrate		1st wash		2nd wash		3rd wash		1st elution		2nd elution		3rd elution	
					Recovery (%)[b]		Recovery (%)		Recovery (%)		Recovery (%)		Recovery (%)		Recovery (%)		Recovery (%)
1st SPE	Water	400 mg	1905.80	197.59	15.04	8.42	0.64	21.67	1.65	46.59	3.55	791.58	60.24	321.49	24.47	0	0
		600 mg		202.88	15.44	15.09	1.15	37.16	2.83	0	0	764.60	58.19	232.80	17.72	56.21	4.28
		800 mg		236.08	17.97	33.16	2.52	28.53	2.17	1.85	0.14	635.86	48.39	378.30	28.79	52.63	4.01
	Ethanol	400 mg	1453.99	27.55	2.55	45.20	4.18	44.35	4.10	44.74	4.13	577.21	53.34	411.34	38.01	7.03	0.65
		600 mg		13.07	1.21	46.59	4.31	49.91	4.61	0	0	646.70	59.76	411.12	37.99	66.34	6.13
		800 mg		10.90	1.01	37.89	3.50	51.98	4.80	0	0	537.72	49.69	305.55	28.24	45.83	4.24
2nd SPE	Water	400 mg	-	180.04	18.89	150.09	15.75	50.18	5.27	28.21	2.96	33.15	3.48	34.71	3.64	7.28	0.76
		600 mg		-	-	-	-	-	-	-	-	-	-	-	-	-	-
		800 mg		192.78	20.23	160.11	16.80	55.14	5.79	34.38	3.61	45.42	4.77	33.80	3.55	29.71	3.12
	Ethanol	400 mg	-	122.41	16.84	116.49	16.02	26.97	3.71	23.88	3.28	3.19	0.44	11.90	1.64	0	0
		600 mg		-	-	-	-	-	-	-	-	-	-	-	-	-	-
		800 mg		133.93	18.42	114.53	15.75	36.94	5.08	22.39	3.08	14.13	1.94	8.24	1.13	2.46	0.34

[a] GAE, gallic acid equivalent

[b] Recovery (%), the percentage of each fraction recovered from original extract solutions

Table. A-6 The total flavonoid content in mandarin peel extracts

	Extraction	Resin Dose	Contents in 5 mL Original Extract Solution (μg RE[a])													
			Original Solution	Filtrate		1st wash		2nd wash		3rd wash		1st elution		2nd elution		3rd elution	
					Recovery (%)[b]		Recovery (%)		Recovery (%)		Recovery (%)		Recovery (%)		Recovery (%)		Recovery (%)
1st SPE	Water	400 mg	1314.02	197.59	15.04	8.42	0.64	21.67	1.65	46.59	3.55	791.58	60.24	321.49	24.47	0	0
		600 mg		202.88	15.44	15.09	1.15	37.16	2.83	0	0	764.60	58.19	232.80	17.72	56.21	4.28
		800 mg		236.08	17.97	33.16	2.52	28.53	2.17	1.85	0.14	635.86	48.39	378.30	28.79	52.63	4.01
	Ethanol	400 mg	1082.07	27.55	2.55	45.20	4.18	44.35	4.10	44.74	4.13	577.21	53.34	411.34	38.01	7.03	0.65
		600 mg		13.07	1.21	46.59	4.31	49.91	4.61	0	0	646.70	59.76	411.12	37.99	66.34	6.13
		800 mg		10.90	1.01	37.89	3.50	51.98	4.80	0	0	537.72	49.69	305.55	28.24	45.83	4.24
2nd SPE	Water	400 mg	-	43.55	3.31	0	0	9.94	0.76	0	0	64.25	4.89	43.07	3.28	62.67	4.77
		600 mg		-	-	-	-	-	-	-	-	-	-	-	-	-	-
		800 mg		59.76	4.55	0	0	0	0	0	0	75.47	5.74	57.36	4.36	41.29	3.14
	Ethanol	400 mg	-	0	0	0	0	16.94	1.57	16.68	1.54	66.01	6.10	51.49	4.76	71.77	6.63
		600 mg		-	-	-	-	-	-	-	-	-	-	-	-	-	-
		800 mg		0	0	11.52	1.06	0	0	5.95	0.55	70.78	6.54	53.49	4.94	75.06	6.94

[a] RE, rutin equivalent

[b] Recovery (%), the percentage of each fraction recovered from original extract solutions

A.2.3.3 Comparison of new and reused resin in separation

Table. A-7 The sugar contents in mandarin peel water extract

	Resin Dose	Sugars	Contents in 5 mL Original Extract Solution (μg)													
			Original Solution	Filtrate		1st wash		2nd wash		3rd wash		1st elution		2nd elution		3rd elution	
					Recovery (%)[a]		Recovery (%)		Recovery (%)		Recovery (%)		Recovery (%)		Recovery (%)		Recovery (%)
New Resin	400 mg	Glucose	9812.03	7620.14	77.66	2272.50	23.16	345.14	3.52	53.79	0.55	127.29	1.30	31.65	0.32	-	-
		Fructose	14127.23	8914.29	63.10	2695.46	19.08	401.70	2.84	74.75	0.53	148.61	1.05	5.64	0.04	-	-
		Sucrose	5586.45	4533.42	81.15	1723.73	30.86	196.75	3.52	45.33	0.81	19.07	0.34	7.88	0.14	-	-
Reused Resin	400 mg	Glucose	9812.03	7773.25	79.22	2449.60	24.97	333.16	3.40	-	-	167.42	1.71	66.43	0.68	-	-
		Fructose	14127.23	8540.77	60.46	2933.98	20.77	390.83	2.77	-	-	174.26	1.23	33.25	0.24	-	-
		Sucrose	5586.45	4411.41	78.97	1882.75	33.70	175.65	3.14	-	-	70.05	1.25	16.56	0.30	-	-

[a] Recovery (%), the percentage of each fraction recovered from original extract solutions

Table. A-8 The total phenolic content in mandarin peel water extract

	Resin Dose	Contents in 5 mL Original Extract Solution (μg GAE[a])														
		Original Solution	Filtrate		1st wash		2nd wash		3rd wash		1st elution		2nd elution		3rd elution	
				Recovery (%)[b]		Recovery (%)		Recovery (%)		Recovery (%)		Recovery (%)		Recovery (%)		Recovery (%)
New Resin	400 mg	1905.80	515.74	27.06	226.96	11.91	95.76	5.02	8.08	0.42	594.86	31.21	268.91	14.11	70.36	3.69
Reused Resin	400 mg		527.16	27.66	315.29	16.54	133.75	7.02	-	-	538.30	28.25	337.54	17.71	139.93	7.34

[a] GAE, gallic acid equivalent

[b] Recovery (%), the percentage of each fraction recovered from original extract solutions

Table. A-9 The total flavonoid content in mandarin peel water extract

	Resin Dose	Original Solution	Contents in 5 mL Original Extract Solution (μg RE[a])													
			Filtrate		1st wash		2nd wash		3rd wash		1st elution		2nd elution		3rd elution	
				Recovery (%)[b]		Recovery (%)		Recovery (%)		Recovery (%)		Recovery (%)		Recovery (%)		Recovery (%)
New Resin	400 mg	1314.02	197.59	15.04	8.42	0.64	21.67	1.65	46.59	3.55	791.58	60.24	321.49	24.47	0	0
Reused Resin	400 mg		240.94	18.34	0	0	0	0	-	-	668.16	50.85	301.61	338.19	147.04	154.18

[a] RE, rutin equivalent

[b] Recovery (%), the percentage of each fraction recovered from original extract solutions

A.2.4 Total phenolic/flavonoid content of autoclaved crude and SPE extracts

Table. A-10 The total phenolic and total flavonoid content in autoclaved mandarin peel crude and SPE extract samples

		Extraction	Crude Extracts		Washing Fractions		Elution Fractions	
				Recovery (%)[b]		Recovery (%)		Recovery (%)
Phenolic Content	Content in 1 g Peel (μg GAE[a])	Water	6173.60 ± 359.29[ns]	100	2969.60 ± 71.86[ns]	51.70	3318.67 ± 83.39[ns]	53.76
	Content in 1 g Peel (μg GAE)	Ethanol	4330.13 ± 114.16[ns]	100	1784.80 ± 200.46[ns]	41.22	2552.00 ± 199.12[ns]	56.15
Flavonoid Content	Content in 1 g Peel (μg RE[a])	Water	3133.70 ± 54.42[ns]	100	264.64 ± 37.68[ns]	8.44	2625.22 ± 82.39[ns]	83.77
	Content in 1 g Peel (μg RE)	Ethanol	1977.10 ± 66.57[ns]	100	196.81 ± 36.57[ns]	9.95	1733.77 ± 29.43[ns]	87.69

[a] GAE, gallic acid equivalent; RE, rutin equivalent

[b] Recovery (%), the percentage of each fraction recovered from crude extracts

Data are presented as means ± SD of triplicate samples. 'ns' represents no significant difference between non-autoclaved (Table. 3-1) and autoclaved samples.

A.2.5 Identification and quantification of polyphenols in mandarin peel extracts

Table. A-11 Identification of phenolic compounds in crude mandarin peel extracts using phenolic composition information from the literature

Peak N°.	Compounds	Sub family	Reference	Retention time (min)	Wavelength (nm)	Detected in mandarin water and ethanol extracts[a)]
1	Gallic acid	Benzoic acid	Xi et al. (2015)	7.0410	280	-
2	Protocatechuic acid	Benzoic acid	Zhang et al. (2014)	11.080	254	-
3	Chlorogenic acid	-	Zhang et al. (2014)	14.078	330	-
4	*p*-hydroxybenzoic acid	Benzoic acid	Zhang et al. (2014)	15.968	254	-
5	Caffeic acid	Cinnamic acid	Zhang et al. (2014)	17.889	330	-
6	Vanillic acid	Benzoic acid	Zhang et al. (2014)	18.525	254	-
7	*p*-coumaric acid	Cinnamic acid	Zhang et al. (2014)	24.716	280	+
8	Eriocitrin	Flavanone	Zhao et al. (2017)	25.066	280	+
9	Rutin	Flavonol	Xi et al. (2015)	25.164	254	+
10	Neoeriocitrin	Flavanone	Zhao et al. (2017)	26.692	280	-
11	Cynaroside	Flavone	Dong et al. (2019)	27.507	330	-
12	Ferulic acid	Cinnamic acid	Zhang et al. (2014)	28.028	330	+
13	Taxifolin	Flavanonol	Zhang et al. (2014)	28.938	280	+
14	Narirutin	Flavanone	Hu et al. (2017)	30.115	280	+
15	Isorhoifolin	Flavone	Nogata et al. (2006)	30.385	330	-
16	Quercitrin	Flavonol	Zhang et al. (2014)	31.790	254	-
17	Rhoifolin	Flavone	Zhang et al. (2014)	31.819	330	-
18	Naringin	Flavanone	Zhao et al. (2017)	31.866	280	-
19	Hesperidin	Flavanone	Hu et al. (2017)	33.416	280	+
20	Neodiosmin	Flavone	Nogata et al. (2006)	34.176	330	-
21	Neohesperidin	Flavanone	Zhao et al. (2017)	35.148	280	-
22	Didymin	Flavanone	Hu et al. (2017)	39.959	280	+
23	Poncirin	Flavanone	Zhang et al. (2014)	40.239	280	-
24	Eriodictyol	Flavanone	Zhang et al. (2014)	40.473	280	+
25	Luteolin	Flavone	Hu et al. (2017)	40.594	330	-
26	Quercetin	Flavonol	Tripoli et al. (2007)	40.750	254	-
27	Apigenin	Flavone	Tripoli et al. (2007)	42.316	330	-
28	Naringenin	Flavanone	Zhang et al. (2014)	42.379	280	-
29	Diosmetin	Flavone	Zhang et al. (2014)	42.797	330	-
30	Isorhamnetin	Flavonol	Brito et al. (2014)	42.973	254	-
31	Hesperetin	Flavanone	Xi et al. (2015)	43.006	254	-
32	Sinensetin	Flavone	Hu et al. (2017)	45.038	330	+
33	Nobiletin	Flavone	Hu et al. (2017)	46.340	330	+
34	Tangeretin	Flavone	Hu et al. (2017)	47.793	330	+

[a)] + represents that the compound was detected in extracts. - represents that the compound was not detectable in extracts.

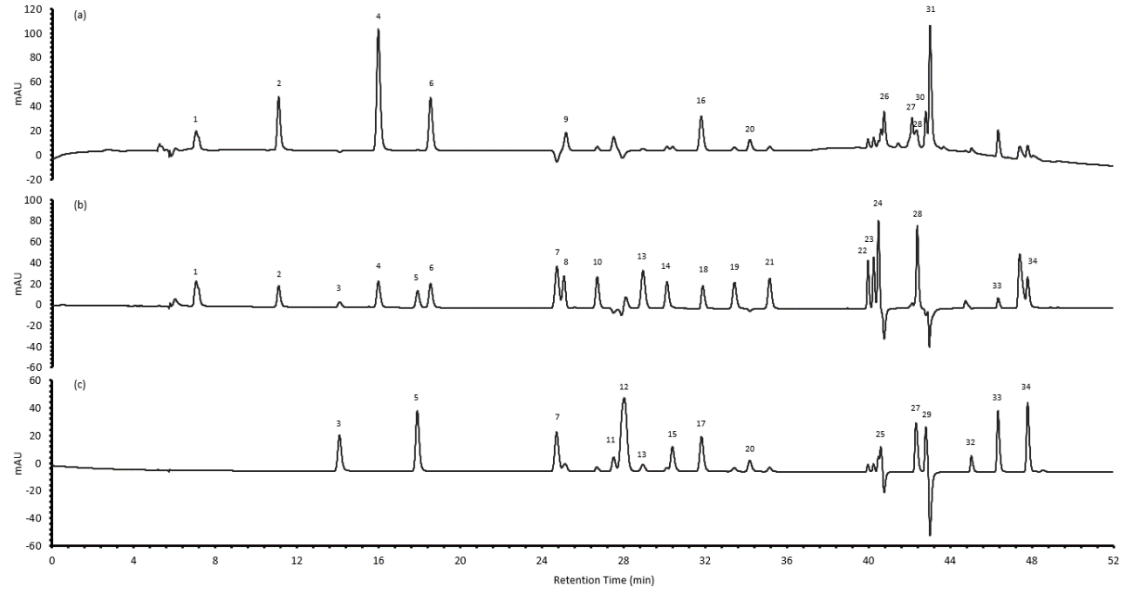

Fig. A-3 HPLC chromatogram of mixed standards at 254 nm (a), 280 nm (b) and 330 nm (c). Peaks: 1, Gallic acid; 2, Protocatechuic acid; 3, Chlorogenic acid; 4, ρ-hydroxybenzoic acid; 5, Caffeic acid; 6, Vanillic acid; 7, ρ-coumaric acid; 8, Eriocitrin; 9, Rutin; 10, Neoeriocitrin; 11, Cynaroside; 12, Ferulic acid; 13, Taxifolin; 14, Narirutin; 15, Isorhoifolin; 16, Quercitrin; 17, Rhoifolin; 18, Naringin; 19, Hesperidin; 20, Neodiosmin; 21, Neohesperidin; 22, Didymin; 23, Poncirin; 24, Eriodictyol; 25, Luteolin; 26, Quercetin; 27, Apigenin; 28, Naringenin; 29, Diosmetin; 30, Isorhamnetin; 31, Hesperetin; 32, Sinensetin; 33, Nobiletin; 34, Tangeretin. The concentration of compound 30 in the mixed standards was 40 μg/mL, and the concentration of other 33 compounds were 10 μg/mL.

Table. A-12 Phenolic composition (µg/g of mandarin peel) of autoclaved crude and SPE extracts with their linear regression equations, correlation coefficients, and limit of detection (LOD) and quantification (LOQ)

Peak N°.	Compounds	Equation A=	r^2	LOD (µg mL^{-1})	LOQ (µg mL^{-1})	Water Extract			Ethanol Extract		
						Crude Extracts	Washing Fractions	Elution Fractions	Crude Extracts	Washing Fractions	Elution Fractions
7	*p*-coumaric acid	53.271x - 5.1516	1.0000	0.39060	0.78125	21.42±1.59 [b]	-	27.43±9.30 [ns]	52.51±7.57 [ns]	-	20.10±0.53 [a]
8	Eriocitrin	31.7x - 2.678	1.0000	0.39060	0.78125	94.69±9.14 [ns]	-	77.03±9.78 [ns]	84.18±4.91 [ns]	-	67.94±4.24 [ns]
9	Rutin	13.506x - 0.2703	0.9998	0.78125	1.56250	217.71±9.37 [ns]	-	196.98±13.50 [ns]	207.34±8.52 [ns]	-	180.44±1.48 [ns]
12	Ferulic acid	53.883x - 6.0778	1.0000	0.39060	0.78125	140.45±6.87 [a]	-	108.40±2.09 [a]	98.07±6.07 [c]	-	83.23±1.02 [c]
13	Taxifolin	48.447x + 1.3298	1.0000	0.39060	0.78125	82.71±4.45 [b]	-	86.94±7.54 [c]	81.17±8.16 [b]	-	80.48±4.77 [c]
14	Narirutin	27.692x - 4.9197	0.9999	0.39060	0.78125	2163.15±18.90 [ns]	13.09±0.00 [b]	2069.08±5.87 [a]	2140.94±12.00 [b]	31.91±0.22 [a]	1828.88±100.04 [ns]
19	Hesperidin	28.402x - 2.3487	1.0000	0.39060	0.78125	1844.23±37.22 [ns]	51.13±0.76 [a]	1551.24±3.19 [c]	1763.96±38.59 [ns]	147.21±2.26 [b]	1426.13±35.35 [c]
22	Didymin	29.655x + 14.594	0.9992	0.39060	0.78125	63.08±3.03 [ns]	-	35.99±6.35 [ns]	45.88±5.25 [ns]	-	43.97±3.79 [ns]
24	Eriodictyol	56.746x - 4.8963	1.0000	0.39060	0.78125	61.61±5.04 [b]	-	50.22±0.50 [a]	60.44±3.62 [b]	-	50.04±1.25 [b]
32	Sinensetin	12.909x - 0.16	0.9999	0.78125	1.56250	82.32±2.85 [ns]	-	77.16±0.77 [ns]	112.27±2.24 [ns]	-	104.27±4.65 [ns]
33	Nobiletin	39.415x - 1.3661	1.0000	0.39060	0.78125	121.01±0.03 [ns]	-	121.10±0.10 [ns]	213.96±0.29 [ns]	-	199.33±7.51 [ns]
34	Tangeretin	49.949x + 0.088	0.9998	0.39060	0.78125	35.39±1.39 [ns]	-	34.79±0.23 [c]	64.82±1.03 [ns]	-	64.36±2.54 [ns]
	Total					4927.77	86.19	4436.34	4925.54	179.12	4149.15

Data are presented as means ± SD of triplicate samples. 'a, b, c' represents significant differences comparing autoclaved samples to non-autoclaved samples (Table. 3-2). a/***, p≤0.0001; b/**, p≤0.001; c/*, p≤0.05; ns, p>0.05.

Table. A-13 Polyphenol content (μg/g) of citrus peel published in the literature

	Literatures	Nogata et al. (2006)	Zhang et al. (2014)	Hu et al. (2017)	Ma et al. (2008)	Hayat et al. (2009)
	Citrus materials	45 citrus genotypes	14 wild mandarin genotypes	*C. reticulata* Blanco cv. Unshiu	*C. unshiu* Marc.	Mandarin (Kinnow)
Family	Compounds					
Flavanones	Hesperidin	0-11700	0-55980	191230	323.54-1077.62	-
	Narirutin	0-3400	0-13610	38020	87.80-296.73	-
	Eriocitrin	0-1560	780-8510	-	-	-
	Neoponcitrin	0-323	-	-	-	-
	Neohesperdin	0-5690	0-11690	-	-	-
	Naringin	0-9940	0-4540	-	-	-
	Poncirin	0-6610	0-2260	-	-	-
	Naringenin	-	0-490	-	-	-
	Taxifolin	-	0-810	-	-	-
	Didymin	-	0-3790	6620	-	-
	Eriodictyol	-	0-460	-	-	-
PMFs	Rutin	0-897	-	-	-	-
	Isorhoifolin	0-510	-	-	-	-
	Diosmin	0-825	-	-	-	-
	Neodiosmin	0-405	-	-	-	-
	Sinensetin	0-3960	70-4710	230	-	-
	Nobiletin	0-5070	510-6830	870	-	-
	Tangeretin	0-3150	190-2920	350	-	-
	Rhoifolin	0-1290	0-3940	-	-	-
	Luteolin	-	0-450	270	-	-
	Diosmetin	-	0-380	-	-	-
	Quercitrin	-	0-860	-	-	-
	3,5,6,7,8,3’,4’-hetamethoxyflavone	-	-	1970	-	-
	5-demethylnobiletin	-	-	210	-	-
Hydroxycinnamic acids	Ferulic acid	-	1613.34-13607.19	-	189.99-1513.21	2054.43-2386.01
	ρ-coumaric acid	-	198.66-3642.63	-	23.00-140.83	425.37-858.40
	Caffeic acid	-	46.24-2055.79	-	12.46-64.28	-
	Sinapic acid	-	0-230.11	-	40.40-132.71	-
Benzoic acids	Protocatechuic acid	-	3.66-48.06	-	15.87-20.68	-
	ρ-hydroxybenzoic acid	-	16.55-95.91	-	14.99-34.11	60.74-72.19
	Vanillic acid	-	23.99-182.81	-	19.23-34.11	196.49-361.53
	Gallic acid	-	-	-	-	79.80-175.22
-	Chlorogenic acid	7.72-61.50	-	-	-	-

- represents not detected.

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