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FULL PAPER

Assessment of toxin-producing genes in *Aspergillus* species from traditional herbal products in Khon Kaen province, Thailand using molecular method

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Abstract

A large number of traditional herbal products from medicinal plants are available in local markets. However, the risk of fungal contamination especially toxigenic *Aspergillus* species, *Aspergillus niger* and *Aspergillus flavus* was potentially possible. Thus, this study targeted to investigate the prevalence of *A. niger* and *A. flavus* and the occurrence of genes encoding toxins, aflatoxins and ochratoxin A among the fungi using the molecular method in 33 herbal products randomly collected from local markets in Khon Kaen province, Thailand. Based on the identification using DNA sequences of the internal transcribed spacer region (ITS), there were two *Aspergillus* species, *A. niger* and *A. flavus,* with prevalence rates of 73.6% and 0.1% respectively. The genes related to aflatoxins and ochratoxin A were undetectable in all isolates. This study suggested the products were naturally contaminated with the fungi, but they were safe from the toxigenic *A. niger* and *A. flavus*.

1. Introduction

Healthcare products from plants are increasingly favourable in Thailand with a high market value, 18,200 million baht in 2019. The products are from the governmental promotion encouraging local people to have their local products called OTOP (One Tambon/ Village One Product) (Kasikornthai Research Centre, 2019). These products are manufactured in different forms of both traditional and processed products available in both national and local markets including Khon Kaen. Some of them are well in the quality control with standard and certified procedures and some are still questionable. One of the quality monitoring methods is to detect fungal strains that possibly produce carcinogenic and deadly toxins e.g., *Aspergillus, Fusarium, Rhizopus, Penicillium*. They produce different forms of aflatoxins, ochratoxin A, citrinin, patulin, and zearalenone (Candlish *et al*., 2001; Ashiq *et al.,* 2014). The most common ones are *Aspergillus flavus and A. niger* found in different products e.g. herbs, spices, tea and coffee (Tournas and Katsoudas, 2008; Aiko and Mehta, 2016). According to the World Health Organization (WHO), materials taken from plants are subjected to the quantification of fungal toxins for consumers' safety (World Health Organization, 2011). The fungal strains are naturally contaminated during the processing and storage (Aquino *et al*., 2010). Therefore, consumers who purchase the products are unable to realize which products contain the toxin-producing fungi. There are standard protocols to detect fungal toxins using analytical chemistry but there are limitations of test sensitivity, cost and time-consuming (Hedayati *et al*., 2007). Additionally, among *Aspergillus* species, they are morphologically similar. It is therefore difficult to determine which strains are *A. flavus* or *A. niger* able to secrete the toxic compounds, aflatoxins and ochratoxin A (Frisvad *et al*., 2011; Pfliegler *et al*., 2020). Thus, a molecular method using specific primers to detect genes encoding the toxins is an interesting tool to overcome this limitation because of its less time consumption and reliability (Geisen, 1996; Sartori *et al.,* 2006; Rodrigues *et al*., 2007; Rahimi *et al*., 2008; Sohrabi and Taghizadeh, 2018). This study accordingly aimed to employ the molecular method to determine whether there were the toxigenic genes in the *Aspergillus* species isolated from the collected herbal products. This could provide rapid detection of the toxigenic fungal strains in the herbal products for customer safety.

2. Materials and methods

2.1 Sample collection

The traditional herbal products were the target of this project. A total of thirty-three samples were randomly collected from local stores and large retailers in Muang district, Khon Kaen province, Thailand. The product

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features were recorded as shown in Table 1.

Table 1. Herbal products collected from the local markets in Muang district, Khon Kaen province, Thailand

No.	Ingredients	Plant parts
1	*Senna alexandrina*	Leaf and pod
2	*Thunbergia laurifolia*	Whole plant
3	*Phyllanthus amarus*	Whole plant
4	*Glycyrrhiza glabra*	Whole plant
5	*Ginkgo biloba*	Leaf
6	*Arcangelisia flava*	Whole plant
7	*Cryptolepis buchanani*	Whole plant
8	*Derris scandens*	Whole plant
9	*Acanthus ebracteatus*	Whole plant
10	*Murdannia loriformis*	Whole plant
11	*Andrographis paniculata*	Whole plant
12	*Orthosiphan grandiflorus*	Whole plant
13	*Momordica charantia*	Fruit
14	*Morus* sp.	Leaf
15	*Senna* sp., *Garcinia cambogia*	Leaf and fruit
16	*Aegle marmelos*	Fruit
17	*Ginkgo biloba*	Leaf
18	*Pandanus amarullifolius*	Leaf
19	*Cymbopogon citratus*	Whole plant
20	*Ganoderma lucidum*	Fruiting body
21	*Cymbopogon citratus* and *Pandanus amarullifolius*	Leaf
22	*Hibiscus sabdariffa*	Fruit and flower
23	*Aegle marmelos*	Fruit
24	*Carthamus tinctorius*	Flower
25	*Stevia rebaudiana*	Leaf
26	*Kaempferia parviflora*	Rhizome
27	*Cantella asiatica*	Whole plant
28	*Vernonia cinerea*	Whole plant
29	*Herbicus sabdariffa*	Flower
30	*Carthamus tinctorius*	Flower
31	*Nymphaea* sp.	Flower
32	*Plukenetia volubilis*	Fruit
33	*Zingiber officinale*, *Ziziphus jujube*, *Tremella* sp.	Fruit

2.2 Fungal isolation

The isolation of fungi was performed by the dilution method. Approximately 1 g of each sample was randomly taken, submerged and agitated for 20 min in 0.1% tween in sterile distilled water. The aqueous suspension was diluted up to 1/10 times and spread onto a standard potato dextrose agar (PDA). The PDA plates were incubated at 25°C for 7 days. All fungal colonies growing on the plates were counted and only *Aspergillus* colonies were isolated. This process was done in 5 replicates.

2.3 Fungal identification

The *Aspergillus* isolates from the previous step were cultured in a broth medium and extracted for genomic DNA as follows. The fungal mycelia on the potato dextrose broth were harvested and washed in sterile distilled water for genomic DNA isolation using the standard method (White *et al*., 1990). Approximately 1 g of the fresh mycelia was finely mashed in liquid nitrogen and 700 µL of the lysis buffer consisting of 25 mM EDTA (pH 8.0), 250 mM NaCl, 200 mM Tris-HCl (pH 8.0) and 2% sodium dodecyl sulfate with 2 µL of ß-mercaptoethanol using the sterile mortar and pestle. Then, the mixture (700 µL) was transferred into new tubes and left for 1 hr at 60°C. Then the mixture, 700 µL of chloroform: isoamyl alcohol (24:1) was added and mixed before being centrifuged at 12,000 rpm for 5 mins at 4°C. The clear supernatant was pipetted to new tubes. Isopropanol, 0.7 times of the supernatant volume was added and incubated at -20°C for 20 mins followed by centrifugation at 12,000 rpm for 5 mins to get the DNA pellets. Then the pellets were washed twice with 70% ethanol, 500 µL and left at room temperature. The dried DNA pellets were dissolved in TE buffer, 50 µL (10 mM Tris-HCl and 1 mM EDTA). RNase A, 2 µL was added and incubated for 20 mins at 37°C followed by 1 µl of Proteinase K with 20-min incubation. To clean the DNA solution, chloroform: isoamyl alcohol (24:1) was added again and centrifuged at 12,000 rpm for 4 mins. The supernatant was carefully taken to new tubes before adding 3 µL of 3M sodium acetate and 150 µL of absolute ethanol and incubated at -20°C for 30 mins. The genomic DNA pellets were then obtained via the last centrifugation at 12,000 rpm for 10 mins. Finally, the pellets were cleaned again with 70% ethanol and left at room temperature until dried before being re-suspended in the TE buffer and stored at -20°C for the next experiment.

In order to identify the randomly selected fungal isolates (Isolate 8-1, Isolate 8-2, Isolate 11-1, Isolate 11-2, Isolate 14-1, Isolate 14-2, Isolate 16-1, Isolate 16-2, Isolate 23-1, Isolate 23-2, Isolate 31-1, Isolate 31-2, Isolate 18-1 and Isolate 18-2), 3 primers were used to amplify the internal transcribed spacer region (ITS), ITS1-TCCGTAGGTGAACCTGCGG, ITS4-TCCTCCGCTTATTGATATGC and ITS5-GGAAGTAAAAGTCGTAACAAGG (White *et al.,* 1990). The condition set for the PCR reaction were: the pre denaturation temperature at 95°C for 3 mins, 35 cycles of 95°C for 1 min and 55°C for 1 min then 72°C for 2 mins and 72°C for 10 mins as the last extension (White *et al.* 1990). One PCR reaction (50 µL) contained autoclaved distilled water, genomic DNA, dNTP mixture, $MgCl_2$, PCR buffer forward primer, reverse

prime and Taq polymerase due to White *et al.* (1990). The positive PCR products were determined in electrophoresis with 1.2% agarose gel under UV. The obtained PCR products were sent for sequencing at 1st Base Company, Malaysia and submitted to MEGA X to identify and remove the ambiguous chromatograms before the phylogenetic analysis. GenBank (www.ncbi.nlm.nih.gov) was the source of the DNA sequences of referencing fungi to compare with the fungal sequences of this study using MEGA X (Table 2).

2.4 Identification of Aflatoxin genes

The genomic DNA of the isolates identified as *A. flavus* derived from the previous experiment was amplified with the primers responsible for Aflatoxin genes as follows; Norsolorinic (nor-1-ACC ACGGCTCCGGCATCGCTCGCA and nor-2-GTTGGCCGCCAGCTTCGACACAGC (Geisen, 1996), Aflatoxin (aflR-F-CGCGCTCCCAGTCTTCCCGATT (Sweeney *et al*., 2000), aflR-R-CTTCCCGTTCGAGATGACCA (Rahimi *et al*., 2008) and omtB-GCCAAATTGCCATGGACATC and omtB-R-CCATGGAGAGCTCCTCTTTA (Rodrigues *et al*., 2007). For *A. niger* isolates, the gene responsible for ochratoxin A production was amplified using RAPDF-CAGTCGTCCAGTACCCTAAC and RAPDR-GAGCGAGGCTGATCTAAGTG (Sartori *et al*., 2006).

The qualitative determination of aflatoxin genes was assessed via the PCR method. The PCR conditions to amplify the *aflR* gene were set according to Rahimi *et al*. (2008), one cycle of initial denaturation at 94°C for 5 mins, followed by 35 cycles of denaturation at 94°C for 1 min, annealing 62°C for 1 min, and extension at 72°C for 2 mins, and a final extension at 72°C for 10 mins. For the *Nor-1* gene, the amplification was optimized according to Geisen (1996), consisting of 30 cycles of denaturation, annealing, and extension at 95°C, 1 min; 65°C, 2 mins, and 72°C, 4 mins, respectively. Another one, *omtB* gene, the protocol was followed due to Rodrigues *et al*. (2007), comprising of an initial denaturation at 94°C for 3 mins, 35 cycles of denaturation, annealing, extension for 1 min 94°C, 1 min 55°C, 1 min 72°C, respectively and 1 cycle final extension for 10 min at 72°C. For ochratoxin A gene, the reaction started with 2 mins at 95°C followed by 35 cycles of 95°C, 1 min; 62°C, 1 min; 1 min, 72 °C and a final extension for 5 mins at 72 °C (Sartori *et al.,* 2006). Different sizes of the DNA fragments on the gel derived from 1.2% agarose gel electrophoresis revealed which the fungal isolates contained the genes, i.e. 593 bp, 365 bp, and 1281 bp for *aflR*, *nor-1*, and *omtB* genes respectively (Pratiwi *et al*., 2015) and the ochratoxin A gene with 372 bp (Sartori *et al.,* 2006).

2.5 Statistical analysis

2.5.1 Prevalence rate

The prevalence rate of the fungal species identified from the collected samples was calculated according to the following equation. Rate of prevalence = (Number of fungal species ×100)/Number of all species

2.5.2 Phylogenetic analysis

The phylogenetic relationship was performed via the Neighbor-Joining method in MEGA X (Saitou and Nei, 1987; Kumar *et al*., 2018). The parameters of the analysis were set as follows. The branch lengths of the trees were in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The replicate trees with associated taxa were grouped in the 1000 bootstrap replicates (Felsenstein, 1985). The p-distance method was used to calculate the evolutionary distances which were in the units of the number of base differences per site (Nei and Kumar, 2013). The complete deletion option was set to eliminate all positions containing gaps and missing data. Evolutionary analyses were conducted and the tree was visualized in MEGA X (Kumar *et al*., 2018).

3. Results and discussion

3.1 Identification of Aspergillus species

In Figure 1, there are two distinct *Aspergillus* species based on their colours, black and green. They were molecularly identified as *A. niger* and *A. flavus* respectively. In Figure 2, the phylogenetic tree using Neighbor-Joining method to indicate the fungal species based on the ITS sequences, the analysis revealed the

Table 2. DNA sequences of *Aspergillus* species and their accession numbers from GenBank

Species	Accession number
Aspergillus flavus	MT529033, MT529482, MT529480, MT529205, MT529008, MN006637, MN006669
Aspergillus niger	MT588793, MT530227, MT316340, MN788116, MN788114, MN788109, MN788108, MN788107, MN585763, MK461093, MK461080,
Aspergillus fumigatus	MT316338, MT297633, MT297629, MK267099, KR023997,
Aspergillus nidulans	MT316339, MT487840, MK806488, MH865286, MH865285, MH864362,
Aspergillus clavatus	HQ026749, AY373847, KF669481, GU183164,
Aspergillus piperis	MT529067, MT529066

optimal tree with the sum of branch length at 0.20850840 with a total of 476 positions in the final dataset and the tree suggested that there were two *Aspergillus* species found in the collected samples, *A. niger* with the bootstrap score at 86 and *A. flavus* (100 bootstrap score).

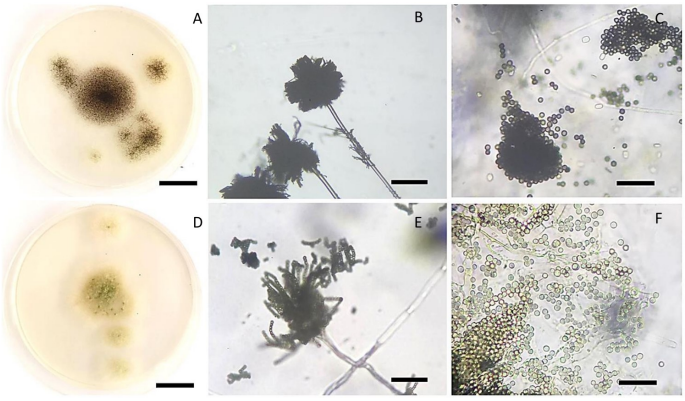

Figure 1. *Aspergillus niger* (A-C) and *A. flavus* (D-F) colonies on the media. Scale bars A and D = 2 cm, B and E = 10 µm, C and F = 5 µm

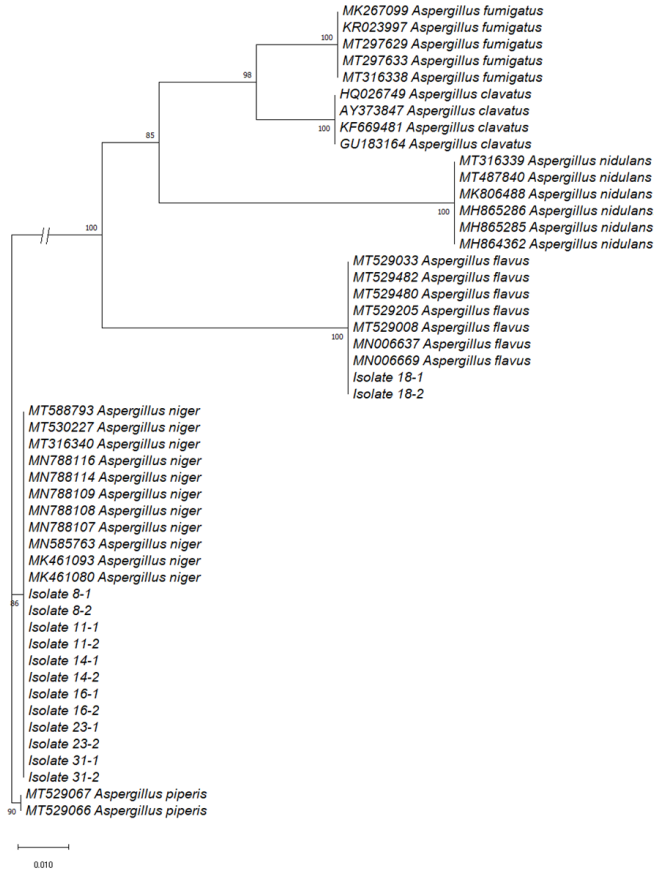

Figure 2. The phylogenetic tree derived from Neighbor-joining analysis indicates the fungal isolates are *Aspergillus niger* with a bootstrap score of 86 and *Aspergillus flavus* supported by a 100 bootstrap score.

3.2 Prevalence of Aspergillus species

Among 33 samples of herbal products, there were 999 fungal colonies. Most of them were *A. niger* (prevalence rate = 73.6%) while there was only one colony of *A. flavus* (Table 3). Based on the finding, *A. niger* was the most prevalent species among the collected herbal products. They are unavoidably contaminated with fungi occurring naturally including *Aspergillus* (Su *et al*., 2018). This group of fungi is commonly found in different food products as well as medicinal herbs with a high prevalence rate, 26.1% (Chen *et al*., 2020). The toxin-producing species, *A. flavus* was also reported to be contaminated in the medicinal herbs and spices (Aiko and Mehta, 2016). Another species, *A. niger* is detected in the products (Su *et al*., 2018). In Brazil, the occurrence rate of *A. niger* isolated from herbal drugs was relatively high, 20.97% (Bugno *et al*., 2006). These species are able to produce the toxins, aflatoxins and ochratoxin A but not all of them were toxigenic (Frisvad *et al*., 2011; Pfliegler *et al*., 2020). Therefore, the identified isolates were amplified for the genes responsible for encoding the toxins.

Table 3. Prevalence of *Aspergillus* species isolated from the herbal products

Aspergillus species	Number of colonies	Prevalence
A. niger	735	0.736 (73.6%)
A. flavus	1	0.001 (0.1%)
Other	263	0.263 (26.3%)
Total	999	999

3.3 Detection of Aflatoxin and ochratoxin genes

The amplification of the toxigenic genes using the mycotoxin-specific primers in both fungal species (*A. niger* and *A. flavus*) was not successful (Table 4). This suggested the genes encoding the toxins were not present in the fungal isolates. However, there are studies reporting the molecular detection of toxigenic genes in *Aspergillus* species. Sohrabi and Taghizadeh (2018) found that 66% of *Aspergillus* species isolated from the animal feedstuff were positive for *aflD (Nor-1)*, *aflP (omt A)* and *aflR* genes. Nagur *et al*. (2014) reported the positive detection of the genes related to aflatoxins production in *A. flavus* derived from peanut products using specific primers, nor-1 (aflD), aflR, omt-1 (aflP) and ver-1 (aflM). Although the aflatoxin genes were negative in the *A. flavus* isolate in this study, this species is also able to produce other toxins like gliotoxin and aflatrem (Hedayati *et al*., 2007). Another species, *A. niger*, was predominantly found in this study and the ochratoxin A production gene was either not detected using the specific primer according to the report by Satori *et al*. (2006). They found that the genes encoding Ochratoxin A were present in some of *A. niger* isolated from the coffee beans using the specific primers. *A. niger* is one of the most common fungi naturally contaminated in food and feed commodities e.g. raw materials for food and feed production (Nielsen *et al*., 2009; Astoreca *et al*., 2010; Marino *et al*., 2014). Although this study did not suggest any positive detection of the toxin genes

encoding aflatoxins and ochratoxin A in the *Aspergillus* isolates derived from the herbal product, there were other unidentified fungal strains, 26.3% that could produce other toxins.

Table 4. Detection of toxin-producing genes from *Aspergillus flavus* and *Aspergillus niger*

Species	Primers			
	nor-1/ nor-2	aflR-F/ aflR-R	omtB-F/ omtB-R	RAPDF/ RAPDR
A. niger	-	-	-	-
A. flavus	-	-	-	-

- not detected, + detected

4. Conclusion

This study found *A. niger* as the dominant fungal species and *A. flavus* as the minority in the collected herbal products. Among them, the genes encoding aflatoxin and ochratoxin A were undetectable using the molecular method. This suggests the products collected from the local market were safe from the toxin-producing fungi. However, there were still other fungal species unidentified that could be harmful. Further studies should therefore target to examine more on this issue in order to identify the fungal strains for their potential effects on the consumers.

Conflict of interest

The authors declare no conflict of interest.

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