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ANTIFUNGAL ACTIVITY OF CLOVE AND BETEL EXTRACT AGAINST *ASPERGILLUS FLAVUS* DURING DRIED CHILI STORAGE

Rungnapa Boonpawa[1*], Fasai Sriyoha[1], and Gavinta Kamnon[1]

[1]*Department of Food Technology and Nutrition, Faculty of Natural Resources and Agro-Industry, Kasetsart University Chalermphrakiat Sakon Nakhon Province Campus, THAILAND*

***Corresponding author**: Rungnapa Boonpawa; rungnapa.boon@ku.th; 59/4, Chiangkrua, Muang district, Sakon Nakhon province, 47000, Thailand

ABSTRACT

Objective: The present study aimed to investigate the antifungal activity of aqueous and ethanol extract of clove bud and betel leaf against *A. flavus* and to explore the possibility of using the plant extract to control the growth of fungi during the storage of dried chili. **Methods**: The antifungal activity of clove bud and betel leaf extract with two extraction solvents, water and 95% (v/v) ethanol, was performed against *A. flavus* TISTR 3151 in PDA. The plant extracts were coated on the surface of dried chili and the fungal growth on dried chili surface were investigated during storage. **Result:** The ethanolic extracts of clove bud and betel leaf exerted 2-5 times higher in antifungal activity against *A. flavus* TISTR 3151 in PDA. A complete inhibition of the fungal mycelial growth was achieved at 10000 ppm ethanolic extract of betel leaf. Soaking dried chili for 30 minutes with ethanolic extracts could reduce the fungal growth up to 3.9 log on day 14. **Conclusion**: Ethanol was shown to be an effective extraction solvent for clove bud and betel leaf for their antifungal activity and the results obtained in the present study point out the possibility of using plant extract to control the growth of pathogenic fungi in dried chili.

Keywords: *A. flavus,* plant extract, antifungal activity, dried chili storage, food safety

1. Introduction

Aflatoxin contamination in food and feed products is remained the most changing food safety problem in Thailand as well as in other countries. Aflatoxins consist of aflatoxin B1, B2, G1, G2 and M1 (metabolized of aflatoxin B1), which aflatoxin B1 is the most toxic as being classified as group 1 carcinogenic agent to humans by International Agency for Research on Cancer (IARC, 2002). Aflatoxin B1 is mainly produced by certain strains of *A. flavus* (Udomkun et al., 2017). Food products especially dried grains, herbs and spices are susceptible to the growth of these pathogenic fungi.

Aflatoxins are heat stable that the temperature as high as 237-306°C is required to decompose the toxins, so normal food processing temperature (80-121°C) could not reduce the amount of aflatoxins contamination in food products (Kabak, 2009). Since it is quite difficult to reduce the amount of contaminated aflatoxins in the products, perhaps alternative solution is to prevent the growth of pathogenic fungi.

In the past years, antifungal activity of plant extracts have been intensively studied against several pathogenic fungi strains. Aromatic plants like clove bud (*Syzygium aromaticum*) and betel leaf (*Piper betle* L.), a common plant in Asian including Thailand) are reported to exert antifungal activity against various fungi strains in culture medium experiments (Viu-Martos et al., 2006, Pinto et al., 2009, Srichana et al., 2009, Ali et al., 2010, Pundir et al., 2010, Rana et al., 2011, Patel and Jasrai, 2013, Singtongratana 2015).

The present study aimed to investigate the antifungal activity of aqueous and ethanol extract of clove bud and betel leaf against *A. flavus* and to explore the possibility of using the plant extract to control the growth of fungi during the storage of dried chili.

2. Materials and Method

2.1. Materials

Fresh betel (*Piper betle* L.) leaves, dried clove buds (*Syzygium aromaticum*) and dried chili pods were

purchased from local market in Sakon Nakhon province, Thailand. Carbendazim (methyl benzimidazole-2-ylcarbarmate) was purchased from U-Ben-F, Thailand. Potato dextrose agar (PDA) was purchased from Himedia, India. Ethanol (95%) was purchased from Univar, Thailand. Sodium chloride (NaCl) was purchased from QReC, New Zealand. Tween 80 was purchased from KC, Thailand.

2.2. Preparation of plant extract

Fresh betel leaves were dried at 50°C for 24 hours using a tray drier. Dried plant materials were ground using a high-speed grinder (HC3000Y, Huangceng, China), stored in plastic bag and kept in a dry and dark place until extraction.

Preparation of plant extracts, 20 g of plant powders were extracted with 100 ml of two different solvents (95% ethanol and water) using a shaker (Unimax 2010, Heidolph, Germany) at a speed of 250 rpm for 24 h. The extraction was carried out at ambient temperature. The crude extract was filtered using Whatman no. 4 filter paper and were reduced to dryness using a rotary vacuum evaporator (BÜchi Rotary model R200, Switzerland) at a temperature of 40°C and a pressure of 120-150 mbar for ethanol extraction or 60-70 mbar for aqueous extraction. The dried crude extracts were weighted and stored in brown glass bottle in a dry and dark place for further experiments. The extraction yield was calculated as follows:

Extraction yield (%) = R/S x 100

where R is the weight of the dried extract and S is the weight of raw plant material before the extraction.

2.3. Preparation of fungal strain

A. flavus TISTR 3151 was chosen as the tested fungi in the present study. *A. flavus* TISTR 3151 was obtained from the Microbial strain bank at Department of Food Technology and Nutrition, Faculty of Natural Resources and Agro-Industry, Kasetsart University Chalermphrakiat Sakon Nakhon Province Campus, Thailand. The fungal strain was cultured on PDA at 30°C for 5 days in an incubator and were maintained and stored at 4°C for further experiments.

2.4. Antifungal activity of plant extract in PDA

The activity of plant extract to inhibit *A. flavus* TISTR 3151 growth in PDA were performed using the poisoned food technique according to the methods described by Sudhakar et al. (Sudhakar et al., 2009) with some modifications. The stock solutions of each plant extract (0, 25000, 50000, 75000 and 100000 ppm) were prepared by dissolving the extract powders in 0.01% tween 80. PDA medium with 10% extract were prepared and sterilized at 121°C, 15 ln/inch2 for 15 minutes. In the Laminar flow (model 0138, Triwork, Thailand), 10 ml of each media was vortex and poured into sterile Petri dishes, allowed to cool and solidify. After the medium completely solid, a mycelial disc of 5 mm in diameter of a 5-day old culture of *A. flavus* TISTR 3151 was aseptically inoculated at a center of the petri dishes. PDA containing distilled water and carbendazim (final concentration of 2500 ppm) instead of the extracts were used as the negative and positive controls, respectively. The inoculated plates were incubated at 30°C for 7 days and the colony diameter was measured. The experiments were carried out in duplicates. The percentage of the mycelial growth inhibition was calculated as follows:

% mycelial inhibition = (Dc-De)/Dc x 100

where Dc is the diameter of *A. flavus* TISTR 3151 colony on the negative control (non-inhibition) and De is the diameter of *A. flavus* TISTR 3151 colony on the plate with inhibitor (plant extracts and carbendazim).

2.5. Antifungal activity of plant extract in dried chili during storage

To study the protective effect of the plant extracts against fungal growth during the storage of dried chili under the addition of *A. flavus* TISTR 3151 inoculum, dried chili pods were sterilized at 121°C, 15 ln/inch2 for 15 minutes. The pods were coated with the plants extract by soaking in the ethanol extract at concentration of 10000 ppm for both plants. This concentration was selected based on the results obtained from Section 2.4. After soaking for 5, 15 and 30 minutes, the pods were dried at 70 °C for 2 hours. The coating solution containing carbendazim at concentration of 2500 ppm and distilled water without the extract were used as the positive and negative control, respectively.

As illustrated in Figure 1, sterile filter paper was placed in sterile Petri dish prior moistening the paper with 1.5 ml of sterile distilled water to raise the moisture levels that suitable for fungal growth. A mycelial disc of 5 mm in diameter of a 5-day old culture of *A. flavus* TISTR 3151 was aseptically inoculated at a centre of the sterile filter paper in the petri dishes. Three coated pods for each soaking time were placed around the inoculum. Three replications were maintained for each treatment. The inoculated plates were incubated at 30°C for 7 and

14 days. The total fungal colony on the surface of the dried chili samples was measured using a plate counting technique. Twenty five grams of dried chili were added to 225 ml of 0.85% (w/v) NaCl and mixed for 2 minutes. A series of sample dilutions were then prepared in 0.85% (w/v) NaCl. 0.1 ml of the dilution were pipetted and evenly distributed onto the PDA surface. The inoculated plates were incubated at 30°C for 3 days and the fungal colonies were counted.

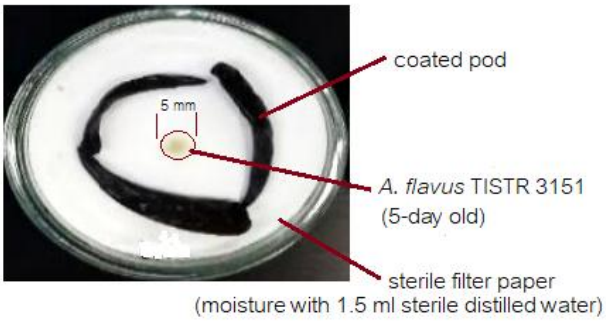

Figure 1: The petri dish layout for the protective effect of plant extracts during dried chilli storage experiment

3. Results

3.1. Antifungal activity of plant extracts against *A. flavus* TISTR 3151 in PDA

The aqueous and ethanolic extract of dried clove bud (20 g) yielded 23 and 34%, respectively. While for 20 g of dried betel leaf, the extraction yield was comparable to that of the clove bud extracts with the aqueous and ethanolic extraction yield of 28 and 38%. The antifungal activity of the plant extracts against *A. flavus* TISTR 3151 in PDA are shown in Figure 2. The results illustrate the dose dependent manner as the concentration of the extracts increased, the more mycelia were inhibited. The antifungal activity of the ethanolic extracts for both plants was found to be 2-5 times more effective than the respective concentration of the aqueous extracts. The ethanolic extracts of betel leaf at a concentration of 7500 ppm could inhibit the mycelial growth by 84% and complete inhibition was achieved at 10000 ppm. While, the ethanolic extracts of clove bud at a concentration of 10000 ppm could inhibit the mycelial growth by 83%.

3.2. Antifungal activity of plant extracts during dried chili storage

The ethanolic extracts of clove bud and betel leaf at a concentration of 10000 ppm were selected to test its antifungal activity against the fungal growth on dried chili surface during storage. The number of fungal colony growth on dried chili surface coated by soaking with ethanolic plant extract for 5, 15 and 30 minutes or coated without the plant extracts is presented in Figure 3. The results showed that ethanolic clove extract with the soaking time of 30 minutes exert the highest antifungal activity resulting in 1.9 and 3.9 log reduction in fungal growth as compared with the negative control on day 7 and 14, followed by the soaking time of 15 minutes the log reduction was 1.9 and 2.1 on day 7 and 14, respectively. While 5 minutes soaking with ethanolic clove extract resulted in the lowest antifungal activity of 0.5 and 1.0 log reduction in fungal growth on day 7 and 14. For ethanolic betel leaf extract, similar trend in antifungal activity as ethanolic clove extract was observed as the soaking time of 30 minutes exert the highest antifungal activity with a value of 1.9 and 2.1 log reduction on day 7 and 14, followed by soaking time of 15 minutes resulting in non-reduction and 1.7 log reduction on day 7 and 14. As for the soaking of 5 minutes, the number of fungal growth was inhibited by 0.2 and 1.2 log reduction on day 7 and 14. Overall, the coating time had an influence on the antifungal activity of the ethanolic plant extracts and the ethanolic clove extract exerted the highest antifungal activity especially on day 14 with the soaking time of 30 minutes.

4. Discussion

Plant extracts might be used as an alternative method as well as a more sustainable solution to control the growth of the pathogenic fungi in food products. The present study aimed to investigate the antifungal activity of aqueous and ethanolic extract of clove bud and betel leaf against *A. flavus* and to explore the possibility of using the plant extract to control the growth of fungi during the storage of dried chili.

The antifungal activity of aqueous and ethanolic extracts of clove bud and betel leaf showed a dose dependent manner, which ethanolic extracts of both plants exerted 2-5 times higher antifungal activity against *A. flavus* TISTR 3151 in PDA as compared with the aqueous extracts. This implies that the active compounds, which are responsible for the antifungal activity in clove bud and betel leaf, are more suitable to extract with ethanol than with water based on the extraction methods described in the present study. Patel and Jasrai found that aqueous extracts of betel leaf (plant powder was extracted with 50ºC distilled water) was ineffective to inhibit the growth of 11 fungi strains, whereas the methanolic extract of betel leaf could inhibit the growth of 10 out of 11 fungal strains including *A. flavus* (Patel and Jasrai et al., 2013).

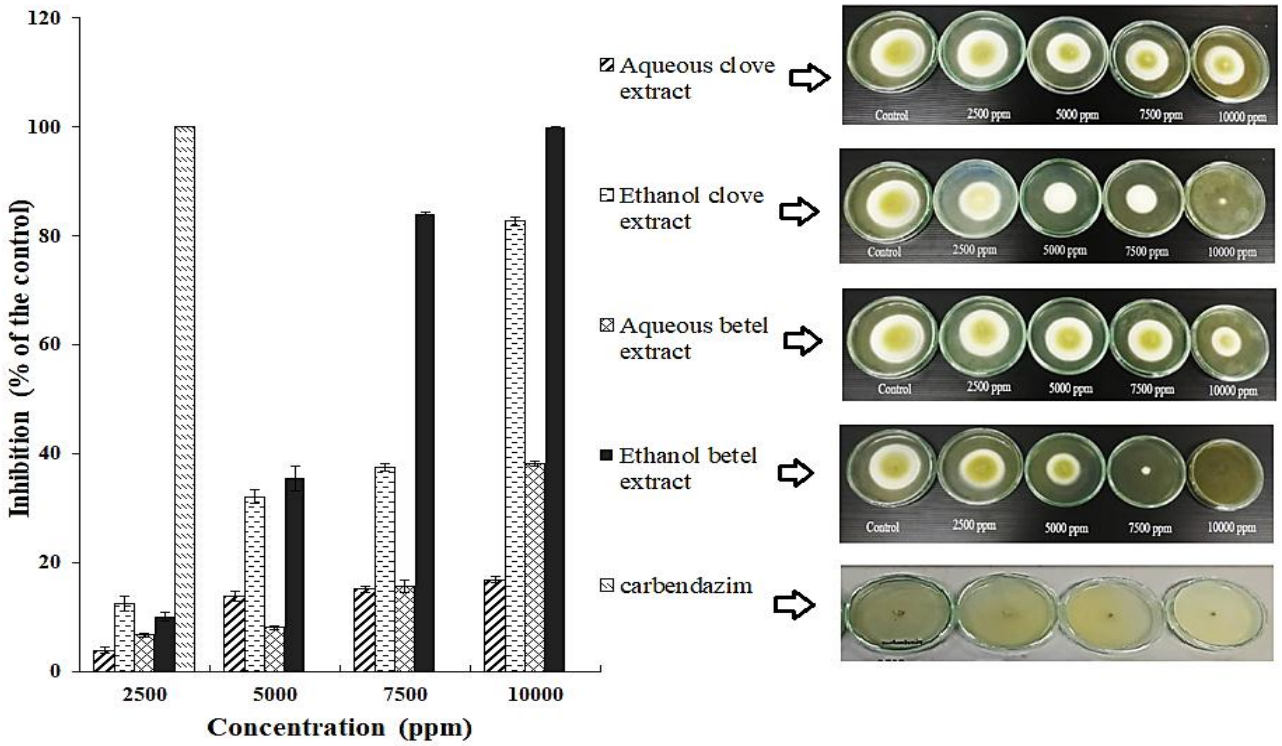

Figure 2: The mycelial growth inhibition activity of plant extracts agains *A. flavus* TISTR 3151 in PDA. Data were expressed as mean ± standard deviation obtained from duplicated experiments.

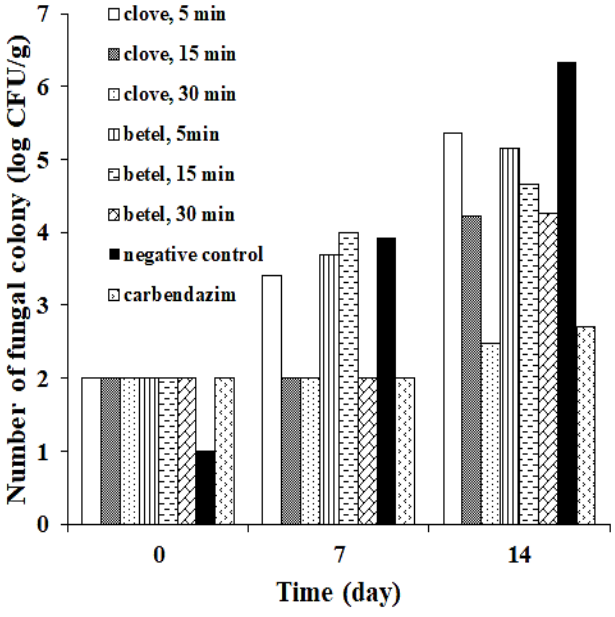

Figure 3: Antifungal activity of ethanolic extracts of clove bud and betel leaf at a concentration of 10000 ppm against fungal growth on dried chili surface coated with ethanolic plant extracts (by soaking for 5, 15 and 30 minutes) and incubated with *A. flavus* TISTR 3151 inoculum at 30°C for 7 and 14 days. The negative and positive controls were the dried chili coated with distilled water and carbendazim, respectively

In the present study, ethanolic extract of clove bud at concentration of 10000 ppm was found to inhibit the fungal mycelial growth of *A. flavus* TISTR 3151 by 83%. This is in line with the reported minimum inhibitory concentration (MIC; defining as the lowest test concentration resulting in complete growth inhibition) for ethanol extract of clove bud against *A. flavus* estimated to be at 20 mg/ml (20000 ppm) (Pundir et al., 2010). While, ethanolic extract of betel leaf at concentration of 10000 ppm could completely inhibit the mycelial growth. Srichana et al. reported similar result that ethanolic extract of betel leaf at a concentration of 10000 ppm was found to completely inhibit the *A. flavus* TISTR 3366 (Srichana et al., 2009). The present study did not identify the active compounds in the ethanolic extract of clove bud and betel leaf.

Based on available data on essential oils, eugenol might be at least partly responsible for antifungal activity in the ethanolic extracts of clove bud as eugenol was previously quantified as the major constituent in clove bud essential oils with the content vary from 48-89% (Lee and Shibamoto et al., 2001, Pawar and Thaker et al., 2006, Chaieb et al., 2007, Pinto et al., 2009, Rana et al., 2011). Eugenol exhibited antifungal activity against a broad spectrum of fungi including *A. flavus*, which the MIC value was reported to be 0.32-0.64 µl/ml (v/v) and its antifungal activity came from its ability to damage the fungal cell membrane by decreasing the amount of ergosterol, a specific fungal cell membrane component (Pinto et al., 2009).

For ethanolic extract of betel leaf, eugenol and hydroxychavicol are likely the major active compounds that are at least partly responsible for antifungal activity in betel leaf extract as betel leaf essential oils contained about 6-20% of eugenol and 24-80% of hydroxychavicol (Ali et al., 2010, Singtongratana et al., 2013, Singtongratana 2015, Preethy et al., 2017, Alighiri et al., 2018). Ali et al. reported that hydroxychavicol isolated from betel leaf could inhibit 124 fungi strains including *A. flavus* by the broth microdilution method (Ali et al., 2010). Besides the antifungal activity against *A. flavus*, ethanolic extracts of clove bud and betel leaf might also inhibit other pathogenic microbes as their essential oils were reported to inhibit a broad spectrum of pathogenic bacteria and fungi such as *S. aureus*, *E. coli*, *Salmonella* sp., *K. pneumoneae*, *A.niger* and *F. verticillioides*, etc (Srichana et al., 2009, Datta et al., 2011, Joseph and Sujatha et al., 2011, Patel and Jasrai et al., 2013).

The present study explored the protective effect of the ethanolic extract of clove bud and betel leaf against fungal growth during dried chili storage by soaking dried chili with 10000 ppm of ethanolic extract of clove bud or betel leaf at different soaking time. The results indicated that the soaking time had an influence on the effectiveness of the ethanolic extracts, which 30 minutes soaking time exhibited the highest efficiency against the fungal growth for 14 days. And the ethanolic extract of clove bud was more effective than ethanolic extract of betel leaf in controlling the growth of fungi during storage. These results were different from the antifungal activity of ethanolic extracts of clove bud and betel leaf in PDA, where at similar concentration ethanolic extracts of betel leaf was more effective in inhibiting the fungal mycelial growth. This might have to do with the stability of the active compounds in the extracts as on day 7 the number of fungal colony were quite comparable for both extracts, however on day 14 the number of the fungal colony in the dried chili coated with ethanolic betel leaf extracts were higher than the clove extract.

5. Conclusion

In conclusion, ethanol was shown to be an effective extraction solvent for clove bud and betel leaf for their antifungal activity and the results obtained in the present study point out the possibility of using plant extract to control the growth of pathogenic fungi in dried chili. However, further study on the toxicity of the plant extract to establish a safe level to use in the food products, the effect of plant extract on food products quality especially the sensory properties as well as technique to incorporate the plant extract in the food products to increase the stability of the active compounds before the plant extract can be fully used as antifungal agent and/or antimicrobial agent in food products.

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