Inhibitory effect of mixed betel leaves and cinnamon crude extracts on growth of *Aspergillus flavus* and *Aspergillus niger* isolates

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Abstract

Different plant essential oils or extracts have often been reported to possess antimicrobial potential. The aim of this study was to evaluate the efficacy of mixed ethyl acetate extracts from dried betel leaf (*Piper betel* L.) and dried cinnamon (*Cinnamomum verum*) on growth inhibition of the fungi, *Aspergillus flavus* and *Aspergillus niger* isolated from natural commodities using disc diffusion method and effect of volatile test. From Disc diffusion test, effectiveness of mixed crude extracts at the concentration range of 5,000-500,000 ppm were determined in Malt Extract Agar (MEA) for 20 days. The results showed that mixed crude extracts from the concentration more than 50,000 ppm could inhibit growth and delay spore development of *A. flavus* and *A. niger* within 7 days. Lower concentration could inhibit the growth of *A. flavus* and *A. niger* for a shorter time. The lowest concentration which could inhibit growth of *A. flavus* was 3,000 ppm. For effect of volatile test, it was found that mixed crude extracts at the concentration of 500,000 ppm had a fungicidal effect. Lower concentration could inhibit the growth and sporulation of *A. flavus* but at a lesser extent.

Keywords: crude extracts, betel leaf, cinnamon, *Aspergillus flavus*, *Aspergillus niger*

Introduction

Fungal contamination is one of a major problems of food and feedstuffs during storage. Moulds have potential to spoil the food stuffs by producing various hydrolytic enzymes. Different types of mycotoxins have been also reported in various edible commodities contaminated by moulds (Tatsadjieu *et al*., 2009). Aflatoxicosis is ranked 6th among the 10 most important health risks identified by WHO (Williams *et al*., 2004). *Aspergillus flavus* is one of the most important mould with the potential to produce Aflatoxin, while *A. niger* is known to be producers of various mycotoxins; ochratoxin A (Noonim *et al*., 2008), fumonisins (Noonim *et al*., 2009). Several chemical additives and preservatives are widely used to prevent post-harvest losses due to fungal contamination. However, many problem related to the development of fungus resistance and health concern have emerging. Aromatic organic compounds of various plants and spices may be an alternative as it possess antifungal activity (Prakash *et al*., 2015). *Piper betle* L.var *magahi* is a climber plant in Indo-Malaya region. Its medicinal application has been well known for many years. Many properties of betel essential oils such as carminative, aphrodisiac and

anticancerous properties have been reported (Manosroi, Dhumtanom and Manosroi, 2006; Bissa, Songara and Bohra, 2007). Cinnamon (*Cinnamomum verum*), is a plant with many uses as spices and herbs, and contains various types of naturally components. Essential oil is the most important, a substantial portion of which is made up of cinnamaldehyde. Cinnamon essential oil and its component cinnamaldehyde have been found to have high antioxidant, antibacterial, and anti-inflammatory activities, and are currently widely used in cosmetic and food industries. Many researchers have reported the inhibitory activity of cinnamon, clove oils against different microbial species (Velluti *et al.*, 2003). Cinnamon and clove oils were found to be effective against aflatoxin production by *A. flavus* (Bullerman, Lieu and Seier, 1977; Sinha, Sinha and Prasad, 1993; Montes-Belmont and Carvajal, 1998). Although the antifungal activities of some essential oils have been well identified, their modes of action toward pathogenic fungi remain largely unknown. Moreover, the effect of mixed essential oils or extracts is not investigated. Therefore, in the present study, preliminary study on the inhibitory effect of betel leaves and cinnamon extracts on growth of *A. flavus* and *A. niger* were assessed. The objective of this study was to assess the inhibitory effect of mixed betel leaves and cinnamon crude extracts on growth of *A. flavus* and *A. niger* isolates.

Materials and Methods

Extraction of crude extracts

Leaves of betel (*Piper betle* L. var *magahi*) and dried cinnamon (*Cinnamomum verum*) powder were purchased from the local market of Surat Thani, Thailand. Betel leaves were cut into small pieces, dried in hot air oven at 70^{o}C for 12 hours, and ground into dried betel powder. Betel powder and cinnamon powder were then soaked in ethyl acetate (1:4 w/v) for 48 hours, then filtered. The solvent was removed by vacuum rotary evaporator. Extract was stored at -18^{o}C for the experimental processes (Adapted from Prakash *et al*. (2010)).

Isolation of fungal strain

A total of 10 samples of peanuts (*Arachis hypogaea*) and shallots (*Allium cepa* var. *aggregatum*) were purchased from retail shops located in Surat Thani, Thailand. The collected samples were stored in sterilized polyethylene bags to prevent further contamination and stored at 8^{o}C until fungal isolation. Direct plating method was used for mycological isolation from peanuts and shallots samples. A total of 50 beans were plated directly (5 particles per plate) onto DG18 and MEA plates. The plates were incubated for 5-7 days at 25^{o}C, and then inspected for mycology growth visually and with the aid of stereomicroscope. Representative colonies of *Aspergillus* isolates in the section *Flavi* and *Nigri* (by morphology used macroscopic and microscopic observation) were isolated and being compared with *A. flavus* BCC 18310 and *A. niger* BCC 17723 purchased from the National Center for Genetic Engineering and Biotechnology, Thailand (Samson *et al*., 2010). Isolates identified as *A. flavus* and *A. niger* were collected and stored for further studies.

Fungal inoculum preparation

From single spore cultures of *A. flavus* grown on MEA media, the spores were harvested from the surface of the agar plates with sterile distilled water. The spore suspensions were filtered through sterile cheese cloth. The concentration of

filtered spores were determined with the aid of haemocytometer and adjusted to a final concentration of 10^5 spores/ml. This spore suspension was used for further evaluation.

Antifungal activity of mixed extracts.

Mixed betel and cinnamon extracts were tested for the abilities to inhibit growth of *A. flavus* and *A. niger* with disc diffusion method (Adapted from 11) and Test-Petri plate method (Adapted from 14). Different concentration of mixed extracts (2,000-500,000 ppm) were dissolved in ethyl acetate. Fungal spore suspension (10^5 spores/ml) in 40% glycerol was prepared for the tests.

Disc diffusion method

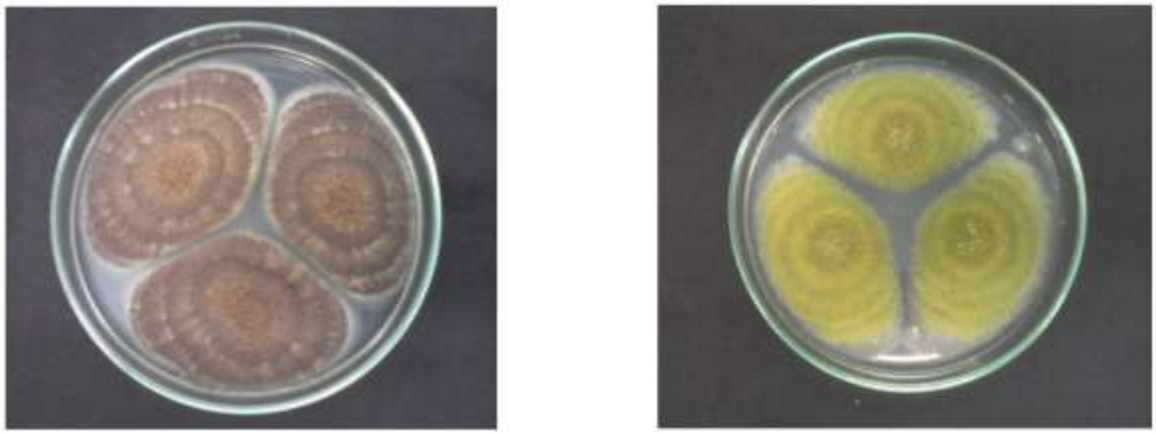
The 6 mm drug testing discs were placed in mixed betel and cinnamon extracts with different concentration and then dried in sterilized plates. Put the prepared drug testing discs onto petri dish spread with fungal spores. Drug testing discs with ethyl acetate were used as control. The plates were then incubated for up to 3 weeks and inspected for the growth of fungi. Three replicates of each sample were investigated. (Adapted from Sindhu *et al*. (2011))

Effect of volatiles

A. flavus and *A. niger* isolates were grown on MEA media as a single colony in the centre of the petri plate. Mixed betel and cinnamon extracts with different concentration (0.1 ml) were poured on the lower lid. For test, the petri plate containing single colony was inverted over the lid containing the mixed extracts, sealed and incubated at 25^{o}C for 21 days. For control, single colony of the fungi alone was placed on the center of MEA media and sealed. Three replicates of each sample were investigated. (Adapted from Sindhu *et al*. (2011))

Results and Discussion

Six *A. flavus* group isolates and 5 *A. niger*group isolates isolated from peanuts and shallots were identified as *A. flavus* and *A. niger* (Figure 1). Three of each were used in this study. Various concentrations of mixed betel leaves and cinnamon extracts were tested for the efficacy to inhibit growth of *A. flavus* and *A. niger* with disc diffusion method (Figure 2) and effect of volatiles test. Mixed betel leaves and cinnamon crude extracts at tested concentrations showed the capacity to reduce or inhibit the growth of both *A. flavus* and *A. niger* isolates. The inhibitory effect of mixed extracts increased proportionally with the concentration. The effect of mixed extracts on inhibition zone of *A. flavus* is presented in Figure 3. The increase in mixed extracts concentration (50,000, 100,000 and 500,000 ppm) caused a dominant delay in growth and spore development within the 7 days incubation period. Lower concentration could inhibit the growth of *A. flavus* for a shorter time. The lowest concentration which could inhibit growth of *A. flavus* was 3,000 ppm but it could inhibit only for 1 day.



A B

Figure 1. Examples of *Aspergillus niger* group (A) and *Aspergillus flavus* group (B) isolated from shallots and peanuts on MEA at 25°C for 7 days and used in this study.

A B

Figure 2. Effect of mixed betel leaf and cinnamon extracts (50,000 ppm) on growth of *A. niger* (A) and *A. flavas* (B) in MEA at 25°C for 5 days by disc diffusion method

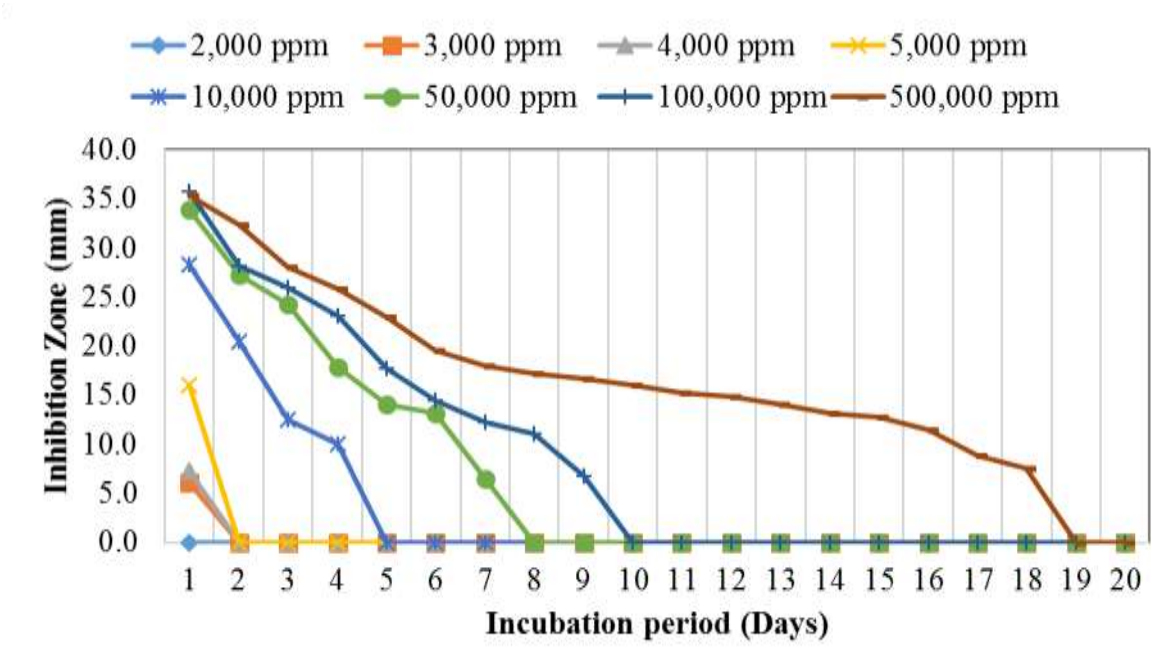


Figure 3. Effect of mixed betel leaf and cinnamon crude extracts at different concentration on growth inhibition of *A. flavus* on MEA at 25°C by disc diffusion method

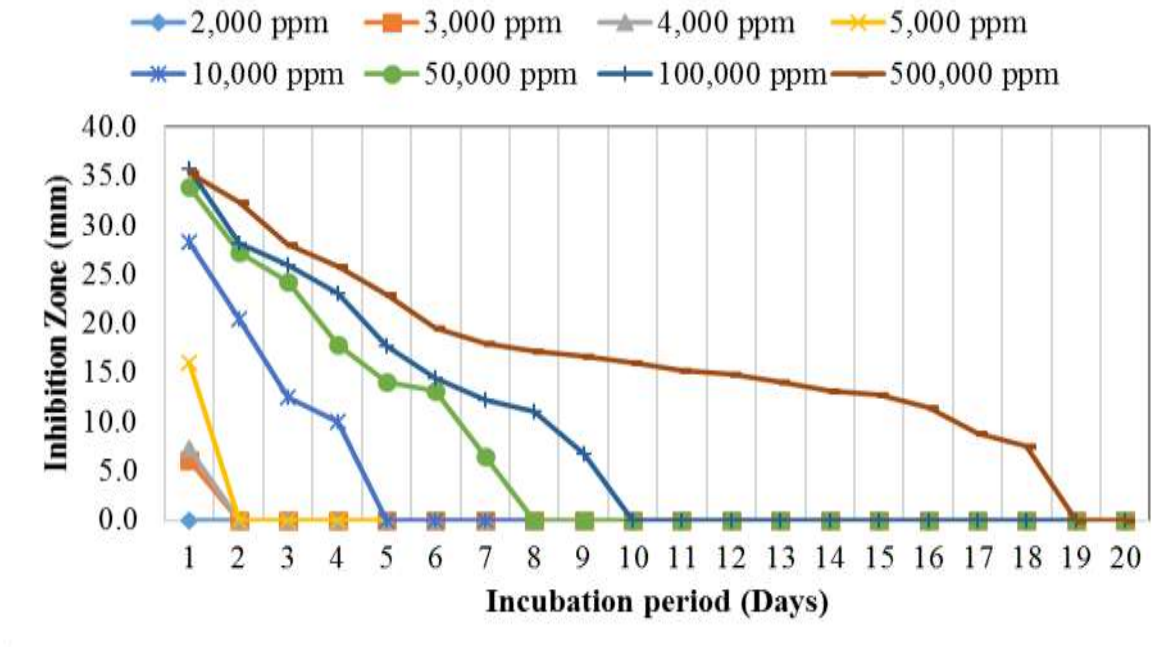


Figure 3. Effect of mixed betel leaf and cinnamon crude extracts at different concentration on growth inhibition of *A. flavus* on MEA at 25°C by disc diffusion method

For *A. niger,* mixed extracts efficacy on inhibition is showed in Figure 4. The similar results were observed. The increase in mixed extracts concentration increased inhibition zone of *A. niger*. Lower concentration could inhibit the growth of *A. niger* for a shorter time. Maximum concentration of mixed crude extracts at 500,000 ppm could delay growth of *A. niger* for 19 days.

The normal methods of testing the antimicrobial activity of natural essential oil or extracts are by supplementing growth media. However, this method does not reflect the actual activity of the volatile fraction. In this study, the experiment on vaporization of the volatile oils against *A. flavus* clearly showed the fungicidal effect. As illustrated in Figure 5, mixed extracts with concentration of 500,000 ppm could totally inhibit growth of *A. flavus.* Lower concentration could inhibit the growth and sporulation of *A. flavus* but at a lesser extent. The similar results were detected with the experiment with *A. niger* (data not shown).

Figure 5. Effect of mixed betel leaf and cinnamon crude extracts volatiles on the growth inhibition and spore production of *A. flavus* cultivated on MEA at 25^{o}C for 5 days; A) 500,000 ppm B) 50,000 ppm

Aromatic organic compounds of various plants and spices possess antifungal activity (5). Recently there has been increasing interest in using naturally occurring compounds, especially essential oils and extracts, to limit fungal growth and toxin production. Theses naturally occurring compounds are known to be quite safe for humans because they have been used as flavoring agents, antioxidant preservative and medicinal herbs (Thanaboripat *et al*., 1997). Fungi inhibiting chemicals have been used for the prevention of fungal growth and preservation of stored products. But as the health concern is a worldwide trend, limiting the use of chemicals in grain and foodstuffs becomes more interested. Natural plant products may be an alternative to these preservatives. Many antifungal compounds derived from plants have been identified (Shelef, 1984, Beuchat and Golden, 1989) against storage fungi for the preservation of spices. Terpenoid and phenolic compounds are two major components of different plant essential oils having lipophilic properties. The lipophilic nature of essential oils appears to play a crucial role for their antimicrobial activity. In generals, antimicrobial essential oils cause structural and functional damages in microbes. Recent GC-MS analysis of betel essential oil revealed 32 different compounds which constitue 97% of the oil. The four major components reported in betel leaves were Eugenol (63.39%), acetyl-eugenol (14.05%) τ-caryophyllene (4.22%) and γ-cadinene (3.85) (Prakash *et al*., 2010). Eugenol was found to be the major and common

compound. However, some earlier works reported phenolics like chavibetol (53.1%) and chavibetol acetate (15.5%) (Rimando *et al*., 1986), safrol (48.69%) (19) and 4-allyl-2-methoxy-phenol acetate (31.47%), 3-allyl-6-methoxyphenol (25.96%) (Apiwat *et al*., 2006) as prime components of *P. betel* essential oils. The efficacy of EO against the moulds is either due to the effect of major component or by the synergistic effect of overall components (Burt, 2004). If compare the results from this study to the earlier work, eugenol was more efficacious as fungal growth inhibitor than essential oil and crude extracts. It reveals that the remaining components of *P. betel* acted in negatively synergistic and reduced the activity of eugenol.

Different species of cinnamon leaves showed the apparent difference in the volatile compound composition of essential oils. However, major constituents in cinnamon leaves and cinnamon barks are cinnamaldehyde (Wang, Wang and Yang 2009; Li, Kong and Wu, 2013). In addition, the bark oils also contained cis-cinnamaldehyde (0.72-2.29%), α-guaiene (1.51-7.62%), copaene (0.58-3.86%), 2-propenal,3-(2-methoxy phenyl) (0.98-2.59%), α-muurolene (0.11-1.83%) and α-calacorene (0.57–1.28%). For *Cinnamomum verum* used in this study, eugenol was also been reported in significant quantity. (23). The antifungal effect of cinnamon oil could be due to several components that are known to have biological activities. The antifungal effects exhibited by the oil or extracts might be due to the effect of the individual constituent and it is also possible that synergistic effects of other minor components may also be responsible for the antifungal effect. Mixed essential oils and extracts can also exhibit positive or negative synergistic effects on growth of fungi.

Conclusion

The present study revealed the efficacy of mixed betel and cinnamon crude extracts as antifungal agent against *A. flavus* and *A. niger*. However some further studies need to be done with more fungal species or different mixture of extracts or essential oils, our study reported possibility of using mixed betel and cinnamon crude extracts as plant based antifungal agent.

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