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INHIBITORY EFFECT OF KAFFIR LIME, BITTER CUCUMBER AND TOBACCO EXTRACTS ON THE GROWTH OF *Aspergillus flavus*

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ABSTRACT

Crude ethanolic extracts of kaffir lime leaf, bitter cucumber fruit and tobacco leaf were examined for their ability to control the growth of *A. flavus* on PDA. The results showed that the ethanolic extracts of all herbs had an inhibitory effect on fungal growth. Kaffir lime at 10 % and tobacco at 8-10 % showed significantly higher inhibition than at other concentrations whereas bitter cucumber at 6, 8 and 10 % gave a similar inhibitory effect on the fungus.

KEYWORDS: herb, medicinal plant, aflatoxin, aflatoxin producing fungi, *Aspergillus flavus*

1. INTRODUCTION

Aflatoxins have been studies extensively because of their high toxicity to certain domesticated animals as well as humans [1-2]. In at least three parts of the world, East Africa, the Philippines and Thailand, good epidemiological evidence has been collected showing a correlation between the incidence of liver cancer and exposure to aflatoxins [3]. Aflatoxins are toxic secondary metabolites produced by certain strains of *A. flavus, A. parasiticus, A. nomius, A. tamarii, A. pseudotamarii* and *A. bombycis* [4-6]. A majority of agricultural commodities are vulnerable to contamination by aflatoxin producing fungi [7]. Aflatoxin producing fungi were found in fermented foods and beverages such as fermented rice, soybean sauce, peanut butter, soy sauce, Thai red and white wine and rice sugar wine [8]. Aflatoxin contamination was shown to be particularly high for low-grade chilies and chili powder sold in supermarkets in India and maize and groundnut samples employed in preparing poultry feed [7]. It was reported that 80% of chili, paprika and other chili products sold in Australia exceeded the aflatoxin contamination limit of 5 ppb [9]. Aflatoxin has been reported to contaminate tobacco [10]. Occurrences of aflatoxin in medicinal plants and spices have also been established [11-12].

Physical, chemical and biological methods have been investigated in order to prevent the growth of aflatoxigenic fungi, eliminate or reduce the toxin levels, and to degrade or detoxify the toxins in foods and feeds. Natural plant extracts are of interest as a source of safer or more effective substitutes for synthetically produced antimicrobial agents and may provide an alternative way to prevent food or feed from fungal contamination [13-15]. Powders and extracts of various herbs, spices and essential oils have been reported to have antimicrobial activity and some also to inhibit aflatoxin formation [16-20].

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Kaffir lime or leech lime (*Citrus hystrix* DC.) and bitter cucumber, also known as bitter melon or balsam pear (*Momordica charantia* Linn.) have been used extensively in traditional Thai medicine. Fruit peel of kaffir lime helps relieve stomach pain whereas extracts of whole bitter cucumber fruit decrease blood glucose concentration. Other properties of bitter cucumber include antifungal, antiparasitic, antiviral, anticarcinogenic *in vivo* [21,22]. A fruit extract demonstrated activity against the stomach ulcer-causing bacteria *Helicobacter pylori*. Tobacco (*Nicotiana tabacum* Linn.) has been shown to be toxic to lice and ticks and tobacco leaves have been successfully used as a protective against plague-infected fleas in India [23]. Certain forms of plant life and bacteria are also susceptible to the poisonous effects of tobacco. In this present study, kaffir lime leaves, bitter cucumber fruits and tobacco leaves were examined for their ability to control the growth of *A. flavus* on PDA.

2. MATERIALS AND METHODS

2.1 Fungal strain

A. flavus IMI 242684 was obtained from the International Mycological Institute, England.

2.2 Herbal extractions

Kaffir lime leaves, bitter cucumber fruits and tobacco leaves were purchased at retail in Bangkok and extracted by the maceration method modified from Nunthavutthikul [24]. The plant materials to be tested were washed and cut into small pieces. One kg of each plant was ground and soaked in 95% ethanol at room temperature for 3 days. The extracts were then filtered through cheesecloth and centrifuged at 3,600 g for 30 mins. The filtrates were then collected, filtered through Whatman No. 1 filter paper and evaporated using vacuum rotary evaporator at 60^{o} C. The dried extracts were collected and kept at 4^{o} C for further investigation.

2.3 Cultivation

Appropriate amounts of extracts were aseptically added into sterile PDA to obtain the final concentrations of 0, 2, 4, 6, 8 and 10%. The medium was then adjusted to pH 3.8-4.0 with tartaric acid. *A. flavus* was point-inoculated on PDA in Petri dish (one colony per dish) and incubated at 25^{o} C for 7 days. Diameter of a single colony of fungus on each Petri Dish was measured every day. All experiments were repeated five times.

2.4 Statistical analysis

The experiments were designed by Randomized Complete Block Design and analysed statistically by the analyses of variance (ANOVA) and multiple comparison at the 1% level [25].

3. RESULTS AND DISCUSSION

3.1 Effect of kaffir lime leaf (KLL) extract on growth of *A. flavus*

The effects of ethanolic extracts of KLL at various concentrations on the growth of *A. flavus* are given in Table 1. The results show that KLL at all concentrations had an inhibitory effect on the fungal growth. The colonial growth was reduced from 89.6 to 61.0, 60.6, 53.6, 53.4 and 41.6 mm on day 7 at concentrations of 2, 4, 6, 8, and 10%, respectively. Statistical analyses showed that this effect was significant at P-value < 0.01(Table2) and multiple comparison test showed that the highest inhibitory effect was at 10% (Table 3).

3.2 Effect of bitter cucumber fruit (BCF) extract on growth of *A. flavus*

The effects of ethanolic extracts of BCF at various concentrations on the growth of *A. flavus* are given in Table 4. The results show that BCF at all concentrations inhibited the growth of

A. flavus. The colonial growth was reduced from 89.6 to 68.8, 63.0, 56.0, 55.8 and 53.8 mm. on day 7 at concentrations of 2, 4, 6, 8, and 10%, respectively. Statistical analyses showed that extracts of BCF significantly inhibited the growth (P-value < 0.01) at all concentrations and multiple comparison test showed that the highest inhibition was at concentrations of 6-10 % (Tables 5 and 6).

Table 1 Effect of ethanolic extracts of KLL on the growth of *A. flavus*

Concentration	Colony diameter (mm.)						
	day 1	day 2	day 3	day 4	day 5	day 6	day 7
0% (control)	20.2	33.0	50.6	60.8	74.0	84.4	89.6
2%	14.0	22.6	32.6	39.4	47.0	56.6	61.0
4%	12.6	20.8	31.2	38.6	47.6	56.0	60.6
6%	11.0	19.8	29.2	36.6	43.4	50.4	53.6
8%	9.2	17.4	25.8	33.0	40.6	48.2	53.4
10%	6.8	11.0	18.8	25.8	31.0	37.2	41.6

Table 2 Analysis of variance for extracts of KLL influencing the growth of *A. flavus*.

Source of variation	Degree of freedom	Sum of Square	Mean Square	F_{cal}	p-value
concentration (treatment)	5	4614.59	922.92	46.15	0.00
day (block)	6	11256.19	1876.03	93.81	0.00
error	30	599.94	19.99		
total	41	16470.73			

Table 3 Multiple comparison test on the effect of ethanolic extracts of KLL at various concentrations on the growth of *A. flavus.*

Concentration	Mean of colony diameter* (mm)
0% (control)	58.94 [a]
2%	39.02 [b]
4%	38.20 [b]
6%	34.85 [bc]
8%	32.51 [c]
10%	24.60 [d]

*Means in the same column with different superscripts are significantly different.

Table 4 Effect of ethanolic extracts of BCF on the growth of *A. flavus*

Concentration	Colony diameter (mm.)						
	day 1	day 2	day 3	day 4	day 5	day 6	day 7
0% (control)	20.2	32.6	50.6	60.8	74.0	84.4	89.6
2%	15.8	23.8	35.8	44.2	53.4	59.6	68.8
4%	15.4	22.8	34.6	40.2	47.6	53.4	63.0
6%	14.6	21.8	33.8	39.4	44.0	51.4	56.0
8%	13.2	21.6	32.8	37.8	42.8	50.4	55.8
10%	12.8	20.8	30.6	36.2	42.0	49.6	53.8

3.3 Effect of tobacco leaf (TL) extract on growth of *A. flavus*

The effects of ethanolic extracts of TL at various concentrations on the growth of *A. flavus* are shown in Table 7. It was found that there was an inhibition of fungal growth at all concentrations. The colonial growth was reduced from 87.0 to 71.2, 50.2, 43.2, 32.0 and 22.6 mm. on day 7 at concentrations of 2, 4, 6, 8, and 10%, respectively. Statistical analyses showed that ethanolic extracts of TL at all concentrations significantly inhibited the fungal growth (P-value < 0.01) and multiple comparison test showed that the highest inhibitory effect was at 8 and 10 %(Tables 8 and 9).

Table 5 Analysis of variance for extracts of BCF influencing the growth of *A. flavus.*

Source of variation	Degree of freedom	Sum of Square	Mean Square	F_{cal}	p-value
concentration (treatment)	5	2752.94	550.58	27.62	0.00
day (block)	6	11472.86	1912.14	95.93	0.00
error	30	597.97	19.93		
total	41	14823.77			

Table 6 Multiple comparison test on the effect of ethanolic extracts of BCF at various concentrations on the growth of *A. flavus.*

Concentration	Mean of colony diameter* (mm)
0% (control)	58.88 [a]
2%	43.05 [b]
4%	39.57 [bc]
6%	37.28 [c]
8%	36.34 [c]
10%	35.11 [c]

*Means in the same column with different superscripts are significantly different.

Table 7 Effect of ethanolic extracts of TL on the growth of *A. flavus.*

Concentration	Colony diameter (mm.)						
	day 1	day 2	day 3	day 4	day 5	day 6	day 7
0%(control)	25.2	39.0	50.8	66.2	79.4	84.2	87.0
2%	15.6	24.4	32.2	41.6	52.0	64.0	71.2
4%	5.0	12.0	17.0	25.4	31.4	42.8	50.2
6%	5.0	8.4	14.4	20.4	27.4	34.8	43.2
8%	5.0	5.0	10.8	18.4	24.6	28.4	32.0
10%	5.0	5.0	5.0	5.0	12.0	18.0	22.6

Table 8 Analysis of variance for extracts of TL influencing the growth of *A. flavus.*

Source of variation	Degree of freedom	Sum of Square	Mean of Square	F_{cal}	p-value
concentration (treatment)	5	12509.28	2501.85	54.50	0.00
day (block)	6	8450.63	1408.43	30.68	0.00
error	30	1377.17	45.90		
total	41	22337.08			

Table 9 Multiple comparison test on the effect of ethanolic extracts of TL at various concentrations on the growth of *A. flavus.*

Concentration	Mean of colony diameter* (mm)
0% (control)	61.68 [a]
2%	43.00 [b]
4%	26.28 [c]
6%	21.94 [cd]
8%	17.74 [de]
10%	10.37 [e]

*Means in the same column with different superscripts are significantly different.

3.4 General discussion

Bitter cucumber contains an array of biologically active plant chemicals including triterpenes, proteins and steroid [26]. The fruit and fruit juice have demonstrated antibacterial properties [22].

Kaffir lime at concentration of 5 and 10 g/100 ml inhibited fungal spore germination of *Colletotrichum gloeosporioides* and *Fusarium* sp., respectively [27]. The ethanolic extracts of kaffir lime leaves inhibited some strains of *Salmonella* [28]. The main compound in kaffir lime leaves is citronellal (65.4 %) whereas the major constituents in essential oil of kaffir lime peels are β-pinene (30.6 %), limonene (29.2 %), and sabinene (22.6 %) [29]. The inhibitory effect on fungal growth of kaffir lime leaf might be due to citronellal.

Zineb and colleagues [30] reported that ethanolic extracts from marine alga, *Cystoseira tamariscifolia*, at the concentration of 10% totally inhibited the growth of *A. flavus* and its mycotoxin formation was inhibited at the concentration of 5% whereas extracts prepared with hexane, methanol and water had no effect on the microbial growth. This may indicate that some of the chemical substances are more soluble in ethanol than other solvents. Ryu and Holt [31] found that spice oils dissolved in soybean oil were less effective in reducing mold growth than when dissolved in water solutions. Ethanolic extracts of olive callus tissues at 0.5 or 1.0% inhibited aflatoxin production by 90% without inhibiting the growth of *A. flavus* [32].

A number of compounds and substances have been found to be effectively inhibit fungal growth and aflatoxin production, while others have stimulatory properties [33]. Microorganisms differ in their resistance to a given herb or spice and the effect of a spice or herb may be inhibitory or biocidal [34]. In many instances low concentrations of test compounds stimulated fungal growth and/or toxin production, while higher concentrations completely inhibited them. Clove oil at 50 and 100 µg/ml and cinnamon oil at 50µg/ml stimulated the growth of *A. flavus* in liquid media whereas higher concentrations reduced the mycelial growth [35].

4. CONCLUSIONS

The antimicrobial activity varies widely, depending on the type of spice or herb, test medium and microorganism [36]. Kaffir lime leaf, bitter cucumber fruit and tobacco leaf extracts have very limited effect on the growth of aflatoxin producing fungus. Water extracts of herbs at low concentrations did not inhibit but rather stimulated the fungal growth. For inhibitory effect, the concentrations of ethanolic extracts were too high for practical application. Thus these herbs are not practical for further use in controlling aflatoxigenic fungi in agricultural commodities.

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