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Inhibition of aflatoxin biosynthesis in *Aspergillus flavus* by phenolic compounds extracted of *Piper betle* L.

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ABSTRACT

Background and Objectives: Food contamination by aflatoxins is an important food safety concern for agricultural products. In order to identify and develop novel antifungal agents, several plant extracts and isolated compounds have been evaluated for their bioactivities. Anti-infectious activity of *Piper betle* used in traditional medicine of Malaysia has been reported previously.

Materials and Methods: Crude methanol extract from *P. betel* powdered leaves was partitioned between chloroform and water. The fractions were tested against *A. flavus* UPMC 89, a strong aflatoxin producing strain. Inhibition of mycelial growth and aflatoxin biosynthesis were tested by disk diffusion and macrodillution techniques respectively. The presence of aflatoxin was determined by thin-layer chromatography (TLC) and fluorescence spectroscopy techniques using AFB1 standard. The chloroform soluble compounds were identified using HPLC-Tandem mass spectrometry technique.

Results: The results, evaluated by measuring the mycelial growth and quantification of aflatoxin B1(AFLB1) production in broth medium revealed that chloroform soluble compounds extract from *P. betle* dried leaves was able to block the aflatoxin biosynthesis pathway at concentration of 500µg/ml without a significant effect on mycelium growth. In analyzing of this effective fractions using HPLC-MS^2 with ESI ionization technique, 11 phenolic compounds were identified.

Conclusion: The results showed that the certain phenolic compounds are able to decline the aflatoxin production in *A. flavus* with no significant effect on the fungus mycelia growth. The result also suggested *P. betle* could be used as potential antitoxin product.

Keywords: phenolic compounds, *A. flavus,* aflatoxin biosynthesis, *Piper betle*, LC-MS^2

INTRODUCTION

Contamination of foods by mycotoxins is an important food safety concern for grains and other agricultural products. Food contaminated with mycotoxins, particularly with aflatoxins, can cause sometimes fatal acute illness, and are associated with increased cancer risk (1). Every year a significant percentage of the world's grain crops are contaminated with hazardous mycotoxins such as aflatoxins. Most countries have established maximum tolerated level for total aflatoxins ranging from 4-20 ng/g (2).

Several methods have been suggested from time to time to prevent the growth of mycotoxin producing fungi, eliminate or reduce the toxin levels, degrade or detoxify the toxins in foods and feeds (3). The use of

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synthetic compounds for control of these fungi have always raised concerns about both the environmental impact and the potential health risks related to their use. In addition to this, synthetic compounds appear to be not very successful against the pathogens (4). Hence, there is a great demand for novel natural antifungal products (5).

Nature has been a source of therapeutic agents for thousands of years and an impressive number of modern drugs have been derived from natural sources (6). Of the roughly 350,000 species of plants believed to exist, one-third of those have yet to be discovered. Of the quarter million that have been reported, only a fraction of them have been chemically investigated (7).

In order to identify and develop novel antifungal agents, several plant extracts and compounds isolated have been examined for their bioactivities. A series of molecules with antifungal activity against different strains of fungi have been found in plants (8). These molecules may be used directly or considered as a prototype for the developing of new medicines (9). The role of oxygenated lipids from polyunsaturated fatty acids (oxylipins) in plants as agents that facilitate resistance to pathogen attach and also toxin biosynthesis were reported by the Christensen and Kolomiets (10). The study on different mycotoxin biosynthetic pathways showed the common factor they share is that they are susceptible to the influence of reactive oxygen species (11). In a review done by Christensen and Kolomiets (10) it was found that some linoleic acid derivatives are able to inhibit toxin synthesis in *Aspergillus* spp.

Several studies have demonstrated significantly higher rate of pharmacological activity in plant extracts used ethnomedically compared to extracts from randomly collected plants (12). Carlson (13) reported that 1.6% of the randomly collected plants were active compared to 15% of the plants presented by the traditional Mayan healer. Over 15,000 flowering plant species were estimated to be found in Malaysia's rainforests (14). In this study we selected *P. betle* which has been used as traditional medicines in Malaysia mainly for treatment of infectious diseases and also cough treatment (15). Anti-infectious effect of *P. betle* against these disease led to the hypothesis that plant-derived phenolic compounds would have an antifungal effect on *Aspergiullus* spp. This hypothesis were supported by studies showing that *P. betle* phenolic compounds exhibit antifungal activities (16).

MATERIALS AND METHODS

Plant materials and extract preparation. *Piper betle* L. was collected from University Putra Malaysia (UPM) agro-farm and voucher specimen (SK 1599/09) was deposited in the biodiversity unit's herbarium, Institute of Bioscience, UPM.

Plant leaves were washed and rinsed in tap water, dried at 40°C using forced air convention oven dryer and powdered by grinder machine. 125 g of sample was macerated at room temperature in 1L of 95% methanol for 24 hours and repeated for three times. The extract was filtered through Whatman No. 1 filter paper and evaporated to dryness under reduced pressure on a Buchi rotary evaporator model R-215 (Switzerland) to provide a sticky crude extract. The crude extract suspended in 90% methanol and subsequently partitioned with an equal volume of chloroform to afford the fraction of chloroform and water (17). The fractions were concentrated in a rotary evaporator followed by a Freezone 6 freeze dryer (Labconco, USA). Concentrated extract was weighed and resuspended in 2% (W/V) dimethyl sulfoxide (DMSO) in distilled water to prepare appropriate concentrations.

Fungus and inocula preparation. The fungus strain used in this study was *A. flavus* UPMC 89, a strong aflatoxin producing strain, obtained from Microbial Culture Collection Unit (UNiCC), UPM. The cultures were maintained on Czapek Dox agar(CDA) slant at 4°C. The fungus was grown on Czapek yeast agar (CYA: CDA+ 0.5% Yeast) at 28°C for 7 days. Spore suspensions were prepared in sterile distilled water containing 0.5% Tween 20 and adjusted to a concentration of 2-3 $\times 10^6$ spores/ml, corresponding to 0.11 to 0.13 absorbance when a Varian Cary Model 50 spectrophotometer set at 530 nm was used (18).

Culture media. The culture media used were CYA and Yeast Extract Sucrose (YES: yeast extract 2%, sucrose 15%) broth (19) for disk diffusion and toxin producing tests respectively. The 25 ml of YES were dispensed in 150 ml conical flasks, the initial pH of the medium adjusted at 6.5 prior to sterilization at 121°C for 15 min.

Inhibition of mycelial growth. Chloroform and water fractions were preliminary tested for inhibitory

effect against *A. flavus* mycelial growth using disk diffusion technique (20). 100µl of spore suspension (3×10^6 spores/ml) were added to the solid CYA medium in 9 cm plates and distributed uniformly using a sterile glass rod aseptically. The extract was dissolved in 5% dimethyl-sulfoxide (DMSO) in water (v/v) to a final concentration of 10, 5, 2.5 and 1mg/ml. Sterile paper discs (6 mm, Whatman, UK) were impregnated with 20µl of extract and placed on the inoculated plates. The plates were incubated at 28°C and diameter of the inhibition zone (mm) around the disk was measured after 48 hours.

Inhibition of aflatoxin biosynthesis. One milliliter of fungus spore suspension were added to 150ml conical flasks containing 25 ml of YES broth containing extract at final concentrations of 1000, 750, 500, 250 and 100 µg/ml. At the end of the incubation period (5 days at 28°C without agitation) the liquid culture was filtered through filter paper (Whatman No. 1), the fungal mycelium washed with distilled water, dried at 50°C for 72 h and dry weight were recorded (21). Toxin extraction was performed by removing 5ml of culture fluid from each flask, filtered through 0.2 µm syringe filter (Pall Acrodisc, USA) and extracted with 5 ml of chloroform (22). The organic phase was collected in 25 ml glass beaker and evaporated to dryness under 40°C in an air circulated oven dryer. The residue was resuspended in 500 µl of methanol and kept in dark glass vial at -20ºC before use.

Detection and quantification of aflatoxins. The detection and qualification of aflatoxin was determined by thin-layer chromatography (TLC) and fluorescence spectroscopy techniques respectively (23). TLC was carried out on a silica gel 60 F_{254} (20×20 cm, Merck) using toluene: chloroform: acetone (15:75:10 v/v) as mobile phases. 20 µl of test samples and aflatoxin standards B1 and B2 (Supelco, USA) with 1 µg/ml concentration were spotted on TLC plates in a TLC tank at room temperature. The developed plates were viewed under UV light at 254 and 365 nm.

Quantitative determination of aflatoxin was estimated using a spectrofluorophotometer RF-5301PC (Shimadzu, Japan). The toxins which earlier extracted from culture fluid were diluted with methanol and concentrations were calculated using a linear calibration curve. Calibration curve was generated with a range of AFB1 standards with concentration of 10–150 ppb (ng/mL). The excitation and emission wavelength were 365 and 435 nm respectively. This method gave a detection limit of 5ppb aflatoxin.The experiment was performed in Randomized Complete Block Design (RCBD). Analysis of variance (ANOVA) was performed on data using the SAS statistic software version 9.1. The mean values were compared by the least significant difference (LSD) test and differences were considered significant when $P < 0.05$.

HPLC-Tandem mass spectrometry ($LC\text{-}MS^2$) analysis of chloroform soluble fraction. For HPLC/UV/MS analyses, the chloroform-soluble fraction of *P. betle*, was filtered through Whatman syringe filters (GD/x 13mm, USA). The extracts were analyzed with a HPLC system consisting of an Agilent (1200 series, Germany) system with a diode array detector set at 280 nm. The injection (20 µl) was performed by an autosampler with an injection needle. The analytical column was a Thermo Hypersil Gold C18 (5µm, 150× 4.6 mm i.d, Thermo scientific, Australia). Analytical separation was achieved at a flow rate of 1 ml/min with the following gradient program: 4 min 95% A, 5%B; 30 min 60% A, 40%B; 38 min 5% A, 95%B; 39 min 95% A, 5%B. Eluent A consisted of water containing 0.5% (v/v) formic acid. Eluent B was acetonitrile containing 0.5% (v/v) formic acid. The temperature of column oven was set to 20°C. A 3200 Q-TRAP mass spectrometer (Applied Biosystems, USA) was connected to the LC via an electrospray ion (ESI) source. The spectrometer was operated in negative mode and the detected mass range was set to 100–800 m/z.

RESULTS AND DISCUSSION

Effect of *P. betle* crude extract against *A. flavus* mycelial growth and toxin biosynthesis. Crude extract, chloroform and water fractions prepared from *P. betle* dried leave did not show any inhibitory effect against *A. flavus* at concentration 1-5 mg/ml in disk diffusion test (result did not showed). However, a significant reduction in aflatoxin B1 production was observed for *P. betle* chloroform fraction.

This fraction at 500µg/ml caused a reduction (91%) in mycelial growth and completely inhibited toxin biosynthesis by *A. flavus* in present experiment. Concentration of 100 µg/ml caused a significant ($p \geq 0.05$) reduction in toxin biosynthesis (69.4%) without any significant effect on mycelial growth (9%) (Fig.

Table 1. Identified compounds in chloroform fraction of *P. betle* using LC-MS^2 at 280 nm.

Peak	RT (min)	Compound	[M-H](m/z)	MS fragment ions(m/z)
1	15.2	Chavibetol	163	133,117
2	18.5	Rutin	609	300
3	21.4	Isomethyl eugenol	177	161, 149
4	21.8	Derivative of Heptanone	723	677, 451, 225
5	23.5	Dihydrochalcone	209	156
6	23.8	Hydroxy acetyl glucoside	352	325, 295, 238, 205, 147
7	23.9	Diethylphenylheptanediol	307	280, 269, 253, 175, 134
8	24.7	Hydroxychavicol	149	121, 119
9	26.2	Derivative of Safrole	205	161, 147
10	32.5	Piperolide	256	255, 227, 198, 171, 143
11	34.9	Ethylsitosterol	443	413, 402, 373, 263

RT: retention time
$[M-H]^-$ -negatively charged molecular ion

1). Many researchers have shown that aflatoxin production is not related to fungal mycelial growth (24).

Yu et al. (25) reported that aflatoxin production in *A. flavus* is closely related to pH and aflatoxin biosynthesis occurs in acidic media. The maximal aflatoxin production in experiment was conducted by Buchanan and Ayres (26) occurred at initial pH of 6, as the initial pH levels <6 favored production of aflatoxins B group, whereas levels >pH 6 favored production of the aflatoxins G group. In the present study the initial pH (pH 6.5) also adjusted to a relatively acidic media to increase biosynthesis of aflatoxin in media.

Identification of phytochemicals using LC-MS^2. In total, 11 compounds were identified in the chloroform soluble fraction based on MS data and λ_{max} at 280 nm. The results obtained from the HPLC-MS^2 are presented in Table 1. The major compounds were presented at RT 23.5 and 24.7 min in HPLC chromatogram. The peak at RT 23.5 min had a $[M-H]^-$ at *m/z* 209 with MS^2 yielding a charged fragment ion at m/z 156. This compound was identified as dihydrochalcone.

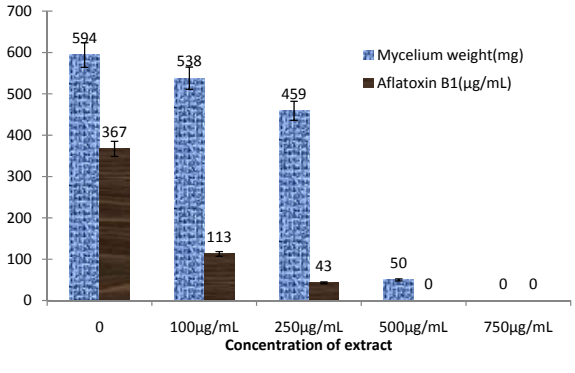

Fig. 1. Effect of *P. betle* chloroform fraction on *A. flavus* mycelial weight and aflatoxin production .

Chalcones and dihydrochalcones were isolated from the *Piper* species (27). Portet (28) isolated and identified the dihydrochalcone from the leaves of *P. hostmannianum* var *berbicense* using MS^2 with APCI in negative mode. The second peak at RT 24.7 min had a $[M-H)^-$ at *m/z* 149, with MS^2 yielding charged fragments ion at *m/z* 121 and 119. This compound was identified as hydroxychavicol. Chavibetol was another phenolic compound product in the extract and was identified through its fragmentation $[M-H]^-$ at *m/z* 163 with MS^2 yielding a charged fragment ion at *m/z* 133 and 117 (Table 1).

Evans (16) isolated hydroxychavicol, chavicol, chavibetol and chavicol acetate with significant fungicidal activity from chloroform extract of the leaves of *P. betle*. In another study, antifungal activities of hydroxychavicol (29) and eugenol (27) extracted from *P. betle* against *A. flavus* were reported.

CONCLUSION

Higher plants have been described as chemical factories that are capable of synthesizing unlimited numbers of highly complex substances (30).

The phytochemicals extracted from *P. betle* inhibited the toxin production via effect on toxin biosynthesis pathway with less effect on fungus mycelial growth.

This result was in concordance with (25) where certain phenolic compounds when added to *A. flavus* media, the aflatoxin production significantly declines causing no effect on fungal growth. Maggon (24) showed that aflatoxin production was not related to fungal mycelium growth. Mode of action of *P. betle* extracts against *Aspergillus* or other fungi is not completely clear yet. The major molecular targets for phenolic compounds present in *P. betle* are proteins (31). Most phenolic compounds (Phenylpropanoides, polyphenols such as flavonoides, catechins, tannins, aquinines) interfere with proteins in an unselective way by forming non–covalent bonds and changing the protein conformation leading mostly to inactivation (32). Hua et al. (33) showed that phenolic compounds considerably retarded biosynthesis of aflatoxin B1 by *A. flavus*. The results confirmed that synthesis and accumulation of norsolorinic acid, an aflatoxin biosynthetic intermediate, was also inhibited by these phenolic compounds.

Dihydrochalcone, hydroxychavicol and chavibetol, the main compounds isolated from P. betle extract in this study, were in accordance with antifungal activities of these compounds which reported (16, 29) previously.

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