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Growth Inhibition of *Aspergillus niger* by Cinnamaldehyde and Eugenol

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ABSTRACT

Inhibitory effects of cinnamaldehyde and eugenol against *Aspergillus niger*, major spoilage mold of fruits and nuts, were investigated. *A. niger* inoculated on agar plates was exposed to paper discs impregnated with 10 μL of cinnamaldehyde and eugenol at various concentrations (10 - 100 μg/ml) before incubating at 25 °C for 3 days. Antifungal activities of cinnamaldehyde and eugenol at the minimum inhibitory concentration (MIC) were examined at various temperatures ranging from 20 °C to 37 °C. The MIC against *A. niger* for cinnamaldehyde and eugenol was 40 μg/mL and 100 μg/mL, respectively. Cinnamaldehyde and eugenol at the MIC completely inhibited *A. niger* up to 14 days within the range of temperature studied. The percentages of mycelium growth inhibition of cinnamaldehyde and eugenol were still higher than 80 % after incubating for 21 and 28 days at all conditions examined. Cinnamaldehyde and eugenol showed good potential as natural antimicrobial additives to inhibit growth of *A. niger* in stored fruits and nuts.

Keywords: Growth inhibition, *Aspergillus niger*, cinnamaldehyde, eugenol

INTRODUCTION

Aspergillus niger is a fungi commonly found on nuts [1], grapes, dried vine fruits [2], apples [3] and tomatoes [4]. *A. niger* can produce ochratoxin A which possess a risk to human health due to the nephrotoxic, immunotoxic, mutagenic, teratogenic and carcinogenic effects [2]. The application of harmless natural preservatives such as substances in essential oils as inhibitors of microorganisms in food products has continued to attract attention in recent years in response to consumer concerns about the uses of artificial chemical preservatives [5]. There have been a number of reports on substances in essential oils that inhibit molds, yeasts and bacteria [6-7]. Cinnamaldehyde and eugenol, major components of cinnamon oil and clove oil, respectively, were reported to inhibit yeasts and molds [8], bacteria [9-10] and spoilage microflora [10-11]. Burt [12] reviewed the possible mechanism of microbial growth inhibition by essential oil components as one or more of degradation of the cell wall, damage to the cytoplasmic membrane, damage to membrane proteins, leakage of cell contents, coagulation of cytoplasm and depletion of the proton motive force.

The objective of this work was to study the inhibitory effects of cinnamaldehyde and eugenol, already registered for uses as food flavourings by the European Commission [12], on growth of *A. niger*.

MATERIALS AND METHODS

Source of inhibitors

Cinnamaldehyde and eugenol were purchased from Sigma-Aldrich Pty. Ltd, Australia.

Fungal culture

The *Aspergillus niger* TISTR 3224 was purchased from the Microbiological Resources Center (MIRCEN), Thailand Institute of Scientific and Technological Research (TISTR), Bangkok, Thailand.

Preparation of inoculum

Spores were obtained from mycelium grown on Malt Extract Agar (MEA) medium incubated at 30 °C for 14 days and were collected by flooding the surface of the plates with ~5 ml sterile saline solution (NaCl, 8.5 g/l water) containing Tween 80 (0.1 % v/v). After counting the spores, the solution was standardized. Fungi growth was observed after inoculation of 10^5 - 10^6 CFU/ml at the center of the plates. Then, a suspension was prepared by adding a loopful of culture to 9 ml of 0.1 % peptone water before use.

Minimum inhibitory concentration (MIC)

The MICs of cinnamaldehyde and eugenol against *A. niger* were determined by the disc diffusion assay. A suspension was prepared by adding a loopful of culture to 9 ml of (NaCl, 8.5 g/l water) containing Tween 80 (0.1 % v/v). Sterile plastic plates of 90 mm diameter (P. Intertrade, Thailand) were used. Sterile paper discs (6 mm Whatman No. 1 filter paper) were impregnated with 10 µL of cinnamaldehyde and eugenol at various concentrations ranging from 10 to 100 µg/mL (i.e. at 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 µg/mL). Different dilutions of cinnamaldehyde and eugenol were made with methanol. Dilution solvent (methanol, 10 µl) was added to the discs on the control plates. The diameter of the zone of inhibition (mm) around the disc was measured after incubation at 25 °C for 3 days.

Mycelium growth inhibition

Antifungal activities of cinnamaldehyde and eugenol were evaluated against *A. niger* by the agar dilution technique of Kuiate *et al* [13]. The 40 µg/mL of cinnamaldehyde and 100 µg/mL of eugenol were incorporated separately into MEA. The control was handled in the same way, but no component was added to the agar. A completely randomized design with six replicates were prepared for each treatment. The diameter of the fungi colony was examined after incubation at 20 °C, 25 °C, 30 °C and 37 °C for 7 days until 28 days and the average value was recorded. The percentage of mycelium growth inhibition was computed after three point inoculation comparison with the control according to the following equation

$$\%inhibition = \frac{dc - dt}{dc} \times 100 \quad (1)$$

where *dc* is the average diameter of fungal colony in the control and *dt* is the average diameter of the fungal colony in the treatment.

Statistical analyses

All variables were tested for normality applying the Komogorov-Smirnov test and homogeneity of variances was assessed using Levene's test. Data transformation was done, where necessary. All results were expressed as a mean ± SD ($n = 6$). The data were statistically treated by one-way ANOVA. Duncan's post hoc tests with $P < 0.05$ were considered to be statistically significant.

RESULTS AND DISCUSSION

Minimum inhibitory concentration (MIC)

Investigation was made on the inhibition of *A. niger* by cinnamaldehyde and eugenol at various concentrations to determine the minimum inhibitory concentration (MIC). Before ANOVA was done, data was transformed with $\log(x+1)$. The MIC against *A. niger* for cinnamaldehyde and eugenol was 40 µg/mL and 100 µg/mL, respectively (**Table 1**). Cinnamaldehyde appeared to be a stronger inhibitor against *A. niger* than eugenol. Reports by other researchers also reveal that cinnamaldehyde strongly inhibits growth of fungi [5,14-17]. Good antifungal activity of essential oil components arises from their hydrophobicity [12]. Phenolic compounds with hydroxyl (e.g. carvacrol) or aldehyde groups (such as cinnamaldehyde) and compounds having a conjugated double bond and a long CH chain outside the ring in essential oil possess strong antifungal activity [12,17-18].

Table 1 Minimum inhibitory concentration (MIC) of cinnamaldehyde and eugenol against *Aspergillus niger* by the disc diffusion assay.

Concentration (μg/mL)	Diameter of inhibition zone ($\bar{X} \pm SD$, mm)	
	Cinnamaldehyde	Eugenol
0	0.00±0.00 [a]	0.00±0.00[a]
10	0.00±0.00 [a]	0.00±0.00[a]
20	0.00±0.00 [a]	0.00±0.00 [a]
30	0.00±0.00 [a]	0.00±0.00 [a]
40	24.83±2.50 [b]	0.00±0.00 [a]
50	31.80±2.29 [c]	0.00±0.00 [a]
60	32.83±1.89 [c]	0.00±0.00 [a]
70	40.50±2.23 [d]	0.00±0.00 [a]
80	42.48±2.12 [d]	0.00±0.00 [a]
90	47.88±1.77 [e]	0.00±0.00 [a]
100	59.33±2.89 [f]	23.56±1.87 [b]

[a-f] Significantly different at the level of $P<0.05$ (column) according to the Duncan test.

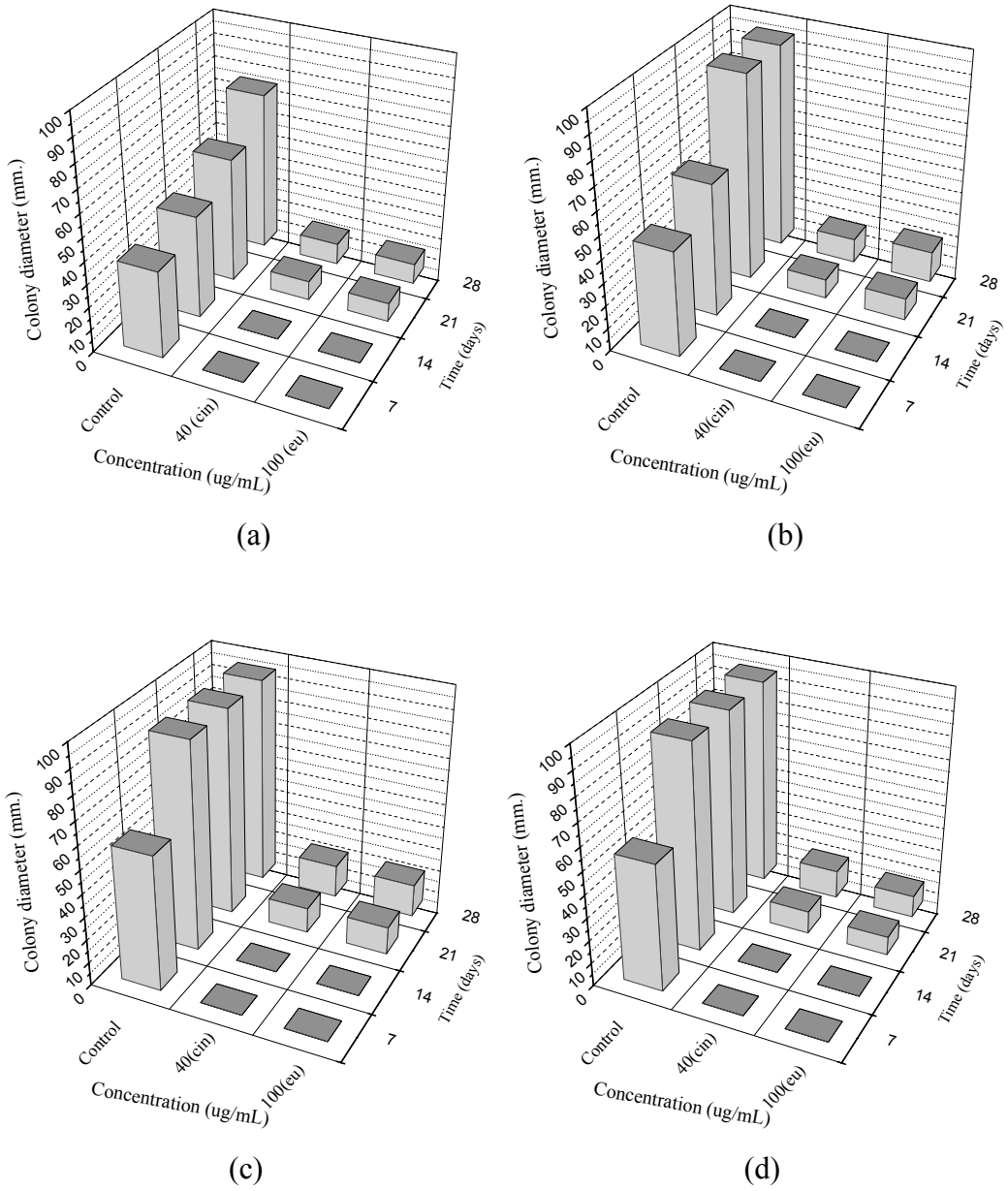
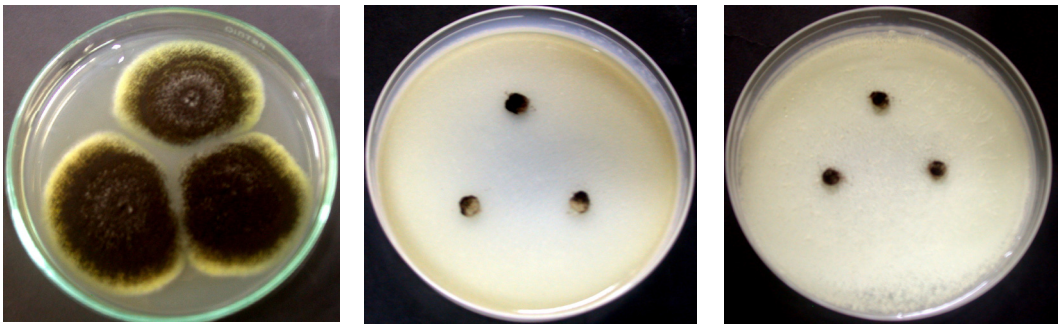


Figure 1 Effects of cinnamaldehyde (cin) and eugenol (eu) at the MIC on growth of *Aspergillus niger* on MEA at (a) 20 °C, (b) 25 °C, (c) 30 °C and (d) 37 °C. The concentration is shown in microgram per millilitre (ug/mL).

Mycelium growth inhibition

The control plates of *A. niger* showed growth after 3 days. The growth of *A. niger* in the control plates (as indicated by the colony diameters) was found to increase with increasing temperature of incubation from 20 °C to 37 °C (**Figures 1a-d**). However, the growth of *A. niger* treated with 40 µg/mL of cinnamaldehyde and 100 µg/mL of eugenol, appeared to be insensitive to temperature. Cinnamaldehyde and eugenol at the MICs (40 µg/mL and 100 µg/mL, respectively) completely inhibited growth of *A. niger* on MEA up to 14 days at all temperature conditions (**Figure 2**).



(a) (b) (c)

Figure 2 Growth of *Aspergillus niger* after 14 days at 20 °C on (a) MEA without essential oil components (b) MEA with 40 µg/mL of cinnamaldehyde and (c) MEA with 100 µg/mL of eugenol.

The percentages of mycelium growth inhibition of cinnamaldehyde and eugenol, examined after 21 and 28 days were higher than 80 % at all temperatures tested. The values were between 85 % - 91 % and 84 % - 89 % for mycelium growth inhibition of cinnamaldehyde and eugenol, respectively (**Figure 3**).

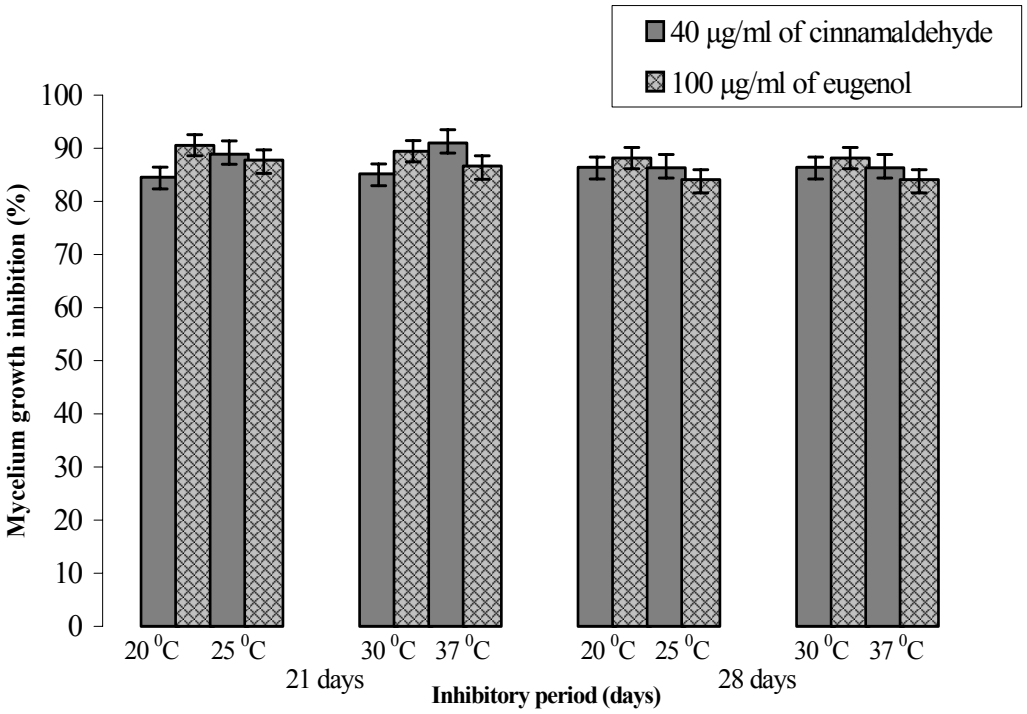


Figure 3 The mycelium growth inhibition (%) of 40 µg/mL of cinnamaldehyde and 100 µg/mL of eugenol against growth of *Aspergillus niger* at various temperatures after 21 and 28 days.

CONCLUSIONS

Cinnamaldehyde and eugenol showed good potential to inhibit growth of *A. niger*. The MIC against *A. niger* of cinnamaldehyde and eugenol was 40 µg/mL and 100 µg/mL, respectively. Cinnamaldehyde and eugenol at the MIC completely inhibited growth of *A. niger* up to 14 days under temperatures of 20 °C - 37 °C. The percentages of mycelium inhibition of cinnamaldehyde and eugenol at the MICs, examined after 21 and 28 days, were between 85 % to 91 % and 84 % to 89 %, respectively. Sensory tests with consumers will also need to be conducted to find a suitable concentration of cinnamaldehyde and eugenol for specific products.

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บทคัดย่อ

นฤมล มาแทน

การยับยั้งการเจริญเติบโตของเชื้อรา *Aspergillus niger* โดยใช้ซินนามาล์ดีไฮด์ (cinnamaldehyde) และยูจีนอล (eugenol)

การศึกษาผลของสารซินนามาล์ดีไฮด์และยูจีนอลในการยับยั้งเชื้อรา *Aspergillus niger* ซึ่งเป็นเชื้อราที่พบได้ทั่วไปในผลไม้และถั่ว การหาปริมาณของสารซินนามาล์ดีไฮด์และยูจีนอลที่ต่ำสุดที่สามารถยับยั้งเชื้อรา *A. niger* นั้นทำโดยการเตรียมสารละลายซินนามาล์ดีไฮด์และยูจีนอลให้มีความเข้มข้น 10 ถึง 100 ไมโครกรัม/มิลลิลิตรในสารละลายเมธานอล จากนั้นจึงดูดสารที่เตรียมไว้มาความเข้มข้นละ 10 ไมโครลิตร เติมลงในแผ่นกระดาษกรองแล้วนำไปวางไว้บนจานอาหารเลี้ยงเชื้อที่มีสปอร์ของเชื้อรา *A. niger* แล้วทำการบ่มที่อุณหภูมิ 25 องศาเซลเซียสเป็นเวลา 3 วัน ผลการทดลองพบว่าปริมาณของซินนามาล์ดีไฮด์ที่ 40 ไมโครกรัม/มิลลิลิตร และยูจีนอลที่ 100 ไมโครกรัม/มิลลิลิตรคือปริมาณของสารที่ต่ำสุดที่สามารถยับยั้งการเจริญของสปอร์ของเชื้อราได้ นอกจากนั้นแล้วซินนามาล์ดีไฮด์ที่ 40 ไมโครกรัม/มิลลิลิตร และยูจีนอลที่ 100 ไมโครกรัม/มิลลิลิตรยังมีฤทธิ์ในการต้านทานการเจริญของเส้นใยของเชื้อราในอาหารเลี้ยงเชื้อได้ถึง 14 วัน ที่อุณหภูมิ 20 ถึง 37 องศาเซลเซียสและเปอร์เซ็นต์การยับยั้งเส้นใยของเชื้อราบนจานอาหารเลี้ยงเชื้อที่ 21 วัน และ 28 วัน ณ อุณหภูมิต่างๆ มีค่าสูงกว่า 80 เปอร์เซนต์ทุกสภาวะ จากการทดลองนี้แสดงให้เห็นว่าซินนามาล์ดีไฮด์และยูจีนอลสามารถยับยั้งการเจริญเติบโตของเชื้อรา *A. niger* ได้และมีความเป็นไปได้ในการนำสารทั้งสองชนิดนี้มาใช้ยับยั้งเชื้อรา *A. niger* ในระหว่างการเก็บรักษาผลไม้และถั่ว

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