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RESEARCH NOTE

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Antibacterial and Sporicidal Activity of Macelignan Isolated from Nutmeg (*Myristica fragrans* Houtt.) against *Bacillus cereus*

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Abstract Macelignan is a bioactive compound isolated from nutmeg (*Myristica fragrans* Houtt.) which has been traditionally used for the food and pharmaceutical purposes. In this study, the activities of macelignan against vegetative cells and spores of *Bacillus cereus* were evaluated *in vitro*. Our results showed that the vegetative cells of *B. cereus* were significantly inhibited in growth by macelignan with minimum inhibitory concentration (MIC) of 4 μg/mL. The vegetative cells of *B. cereus* were completely killed with minimum bactericidal concentration (MBC) of 8 μg/mL of macelignan. Killing time of macelignan against vegetative cells of *B. cereus* was very fast; endpoint of macelignan was reached after 4 hr of incubation at 4×MIC. Macelignan inactivated more than 3-log (99.9%) of spores/mL of *B. cereus* at the concentration of 100 μg/mL. Macelignan was found to be effective against vegetative cells and spores of *B. cereus*. These results suggest that macelignan might be good to be developed as a food preservative.

Keywords: antibacterial, *Bacillus cereus*, macelignan, sporicidal, nutmeg

Introduction

Bacterial spores have special significance in foods because they are much more resistant to physical and chemical antimicrobial treatments. Many species of spore forming bacteria, especially *Bacillus* and *Clostridium*, are associated with food spoilage and foodborne diseases (1). *Bacillus cereus* is a spore-forming bacterial foodborne pathogen, which is ubiquitous in nature, and hence occurs frequently in a wide range of food raw materials (2). The organism is associated with two types of gastrointestinal disorders: the emetic syndrome, characterized mainly by vomiting and caused by ingestion of a preformed toxin in the food, and the diarrhea syndrome, caused by a different toxin that can be formed in the food but also in the small intestine. *B. cereus* is recognized as a leading cause of bacterial food poisoning in several countries, with a variety of proteinaceaous and starchy foods, including rice, milk, meats, souses, and desserts (3,4).

Several food preservation systems such as heating, refrigeration, and addition of chemical antimicrobial compounds can be used to reduce the risk of outbreaks of food poisoning, however, these techniques frequently have associated adverse changes in organoleptic characterizations and loss of nutrient (5). Moreover, consumer demand of natural, fresh, chemical-additive free, and safe food products is increasing (6). Therefore, there is interest recently in development of natural food preservation and processing. Enzymes (lactoperoxidase, lactoferrin, avidin, and lysozyme), microbial preservatives isolated from starter cultures (nisin, lactasin, and variacin), and plants resources are available as natural antimicrobial compounds for use in food preservation and processing (7). Edible medicinal plants are used widely in food industry as flavors and fragrances, also exhibit useful antimicrobial properties (8). Many plant-derived antimicrobial compounds have a wide spectrum of activity against foodborne bacteria and this has led to suggestions that they could be used as natural preservatives in foods (9,10).

Macelignan is a bioactive compound isolated from nutmeg (*Myristica fragrans* Houtt.) which has been traditionally used for the food and pharmaceutical purposes. Macelignan has been reported to have antioxidant, anti-inflammatory, anticariogenic, hepatoprotective, and antibiofilm activities (11-14). Moreover, macelignan did not present any cytotoxic effect at concentrations ranging from 0.5 to 5 μM against HepG2 cells (12). However, antibacterial activities of macelignan against foodborne pathogen such as *B. cereus* have not yet been investigated. Gastrointestinal-upset and vomiting are caused by enterotoxin produced during vegetative growth of *B. cereus* and the spore of *B. cereus* is an important factor in foodborne illness (15). Comparatively few antimicrobial agents isolated from plants extract are actively sporicidal and even quite powerful bactericides may only inhibit spore germination, outgrowth, or both; that is, they are sporostatic rather than sporicidal. Alternative for comparison using chemical agents, the most important chemical sporicides are glutaraldehyde, formaldehyde, chlorine-releasing agents, peroxygens, ethylene oxide, and ozone (16). Thus, the aim of this study was to evaluate the activities of macelignan against vegetative cells and spores of *B. cereus* and glutaraldehyde was used as a control for comparison.

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Materials and Methods

Bacterial strain and inoculum preparation *Bacillus cereus* ATCC 21772 was obtained from the American Type Culture Collection (Rockville, MD, USA). *B. cereus* was cultured in nutrient broth (NB; Difco, Spark, MD, USA) or NB supplemented with 1.5%(w/v) agar (NA) and in Muller Hinton broth (MHB; Difco, Franklin Lakes, NJ, USA) or MHB supplemented with 1.5%(w/v) agar (MHA). A standardized inoculum of *B. cereus* was prepared as follows: each the strain was propagated in MHB at 37°C for 12 hr with 200 rpm agitation. One mL of 12 hr old culture in MHB was centrifuged (4,000×g at 4°C for 1 min), and the pellets were washed twice with 1 mL of physiological saline. Sterile physiological saline was added to give a McFarland turbidity of 0.5 at 530 nm, corresponding to 5×10^6 CFU/mL (17).

Spores preparation *B. cereus* spores were prepared using the method described previously by Kida *et al.* (18, 19) with modification. Briefly, *B. cereus* was grown on NA at 30°C for more than 1 week. After harvesting, spores and vegetative cells were suspended in sterile 0.85% NaCl solution, and incubated at 65°C for 30 min to kill vegetative cells. Spores were harvested by centrifugation and washed 4 times with the original volume of sterile 0.85% NaCl solution by centrifugation (13,000×g for 30 min at 4°C). One mL portion of the spore suspension containing approximately 10^9 spores/mL in a 1.5 mL plastic cryopreservation tube was stored at −85°C prior to use.

Macelignan and glutaraldehyde preparation Nutmegs (*Myristica fragrans* Houtt.) were obtained from Biopharmaca Research Center of Bogor Agricultural University in Indonesia. Macelignan (Fig. 1) was isolated from ethyl acetate fraction of nutmeg using the method described previously (13). Macelignan was dissolved in 10% dimethyl sulfoxide (DMSO) to obtain 1,000 μg/mL stock solution. DMSO at 10% was found not to kill *B. cereus*. A commercial glutaraldehyde (Merck, Darmstadt, Germany) was used as a positive control for sporicidal activity experiments. The glutaraldehyde solution was prepared using a standard 25% commercially available solution (Merck).

Susceptibility test *In vitro* susceptibility tests were performed to evaluate minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of macelignan against vegetative cells of *B. cereus.* MIC and MBC were evaluated using the method described in the guidelines of CLSI M7-A6 (17). Briefly, MIC was determined with the adjusted inoculum suspension of 5×10^6 CFU/mL by diluting 1:10 with MHB medium to make final inoculum concentration of 5×10^5 CFU/mL. Macelignan was diluted 1:10 in MHB medium containing 5×10^5 CFU/mL inoculum, yielding an initial inoculum of 4.5×10^4 CFU/mL. The final concentration of macelignan ranged from 0.25-512 μg/mL. A 200 μL aliquot of each suspension was placed in 96-wells round-bottom microtitration plates. The plates were incubated at 35-37°C, and endpoints were read visually after 24 hr. The MIC was interpreted as the lowest concentration of macelignan totally inhibiting the growth of *B. cereus* compared with the growth control, after the preset incubation time. Assays were performed 3 times with duplicate/experiment. MBC was determined as outlined for MIC by removing the medium from each well showing no visible growth and subculturing onto MHA plates. The plates were incubated at 35-37°C until growth was seen in the growth control plates.

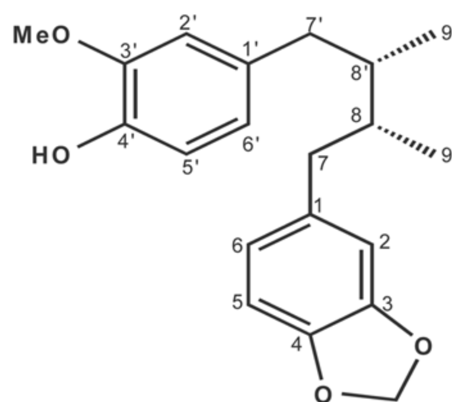

Fig. 1. Structure of macelignan.

Time-kill curve analysis Time-kill curve were performed in MHB medium (17). Each concentration of macelignan was diluted with 1:10 in MHB medium containing 5×10^6 CFU/mL of *B. cereus*. This procedure yielded an initial inoculum 4.5×10^5 CFU/mL. Final concentration of macelignan were 0×MIC, 1/4×MIC, 1/2×MIC, 1×MIC, 2×MIC, 4× MIC, and 8×MIC. Cultures (5 mL final volume) were incubated at 35-37°C with agitation (200 rpm). At predetermined time points (0, 4, 8, 12, 16, 20, and 24 hr), a 100 μL aliquot was removed and transferred to eppendorf tubes, centrifuged (3,900×g at 4°C for 1 min), and rinsed twice with 0.9 mL of sterile distilled water to obtain macelignan-free cells. Pellets were suspended in 100 μL of sterile distillated water and serially diluted. An appropriate volume (100, 40, or 20 μL), depending on the dilution and the concentration of macelignan, was spread onto MHA plates and incubated at 35-37°C for 24 hr or more (until the colonies were seen on the plates) to determine the numbers of CFU/mL. The experiment was repeated 3 times with duplicate/experiment.

Measurement of sporicidal activity Sporicidal activity was determined basically as described previously (18-20), with modification. Briefly, macelignan and glutaraldehyde were serially diluted with 0.85% NaCl solution (pH 6.6) to generate a series of concentrations ranging from 10-1,000 μg/mL. Prepared spores suspension (10^9 spores/mL) was thawed and diluted 1:100 in 0.85% NaCl solution (pH 6.6), yielding an adjusted spores suspension of 10^7 spores/mL. Individual series concentrations of macelignan or glutaraldehyde were diluted 1:10 in adjusted spores suspension, resulting in an initial spore suspension of 10^6 spores/mL. The final concentration of macelignan or glutaraldehyde ranged from 1-500 μg/mL. The pH of these test solutions was not changed by addition of macelignan or glutaraldehyde. One mL of each test solutions were then

exposed for various incubation times in a water bath (30°C). At predetermined time points (0, 0.5, 1, 2, 3, 4, and 5 hr), a 100 μL aliquot was removed and transferred to eppendorf tubes, centrifuged (12,000×g at 4°C for 5 min) and rinsed twice with 0.9 mL of 0.85% NaCl solution (pH 6.6) to obtain antibacterial-free spores and to avoid an effect of vegetative cells residue. Pellets were suspended in 100 μL of 0.85% NaCl solution (pH 6.6) and serially diluted. An appropriate volume (100, 40, or 20 μL), depending on the dilution and the concentration of antimicrobial, was spread onto NA plates and incubated at 35-37°C for 24 hr or more (until the colonies were seen on the plates). Colonies formed were counted and the mean of CFU/mL was calculated. Differences were obtained by subtracting the log CFU/mL values of the test solution from those of the control (no antimicrobial). The mean value and standard deviation (SD) were calculated using differences from 3 independent experiments, and the reduction of spore cells in CFU was expressed as sporicidal activity.

Results and Discussion

Susceptibility test The vegetative cell growth of *B. cereus* was significantly inhibited by macelignan with MIC of 4 μg/mL. There are a few reports concerning the susceptibility of *B. cereus* to a single compound of antimicrobial isolated from plants. Ultee and Smid (21) reported that carvacrol isolated from oreganum (*Oregon geranium*) reduced the viable count of *B. cereus* vegetative cells with concentration of 60 μg/mL. Moreover, the growth of *B. cereus* vegetative cells were inhibited by morin, quercetin, galangin, kaempherol, myricetin, fisetin, naringenin, and eriodictyol with MIC of 300, 350, 800, 800, 250, 400, 50, and 100 μg/mL, respectively (22). Our study showed that macelignan inhibited the growth of vegetative cells of *B. cereus* with much lower MIC against vegetative cells of *B. cereus*. This result suggests that macelignan has strong bacterial static activity against *B. cereus* vegetative cells. *In vitro* MBC of macelignan with endpoint after 24 hr demonstrated that macelignan was able to kill the vegetative cells of *B. cereus* with MBC of 8 μg/mL. This result indicates that macelignan has strong bactericidal activity against vegetative cells of *B. cereus*. To our knowledge, this is the first report of antibacterial activity of macelignan against vegetative cells of *B. cereus*.

Time-kill curve The killing activity of macelignan on vegetative cells of *B. cereus* is presented in Fig. 2. The bactericidal activity of macelignan was fast acting against vegetative cells of *B. cereus*; the reduction in the number of CFU/mL was >3 log units (99.9%). The bactericidal endpoint of macelignan was reached after 8 hr of incubation at 2 ×MIC (8 μg/mL). Moreover, endpoint of macelignan was also reached faster, after 6 and 4 hr of incubation, at concentration of 4×MIC (16 μg/mL) and 8×MIC (32 μg/mL), respectively (Fig. 2). Fast acting killing of an antimicrobial agent against vegetative cells of spore-forming bacteria is very important (23). *B. cereus* is known for the production of spores that allow survival of the organism under severe nutrient deprivation and dehydration conditions. In contrast, spores are even resistant to the actions of common antimicrobial agents and treatments.

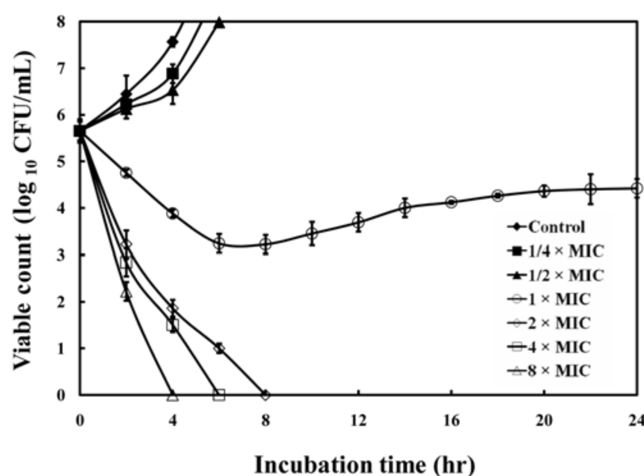

Fig. 2. Time-kill curves of macelignan against vegetative cells of *B. cereus* ATCC 21772 at different concentrations.

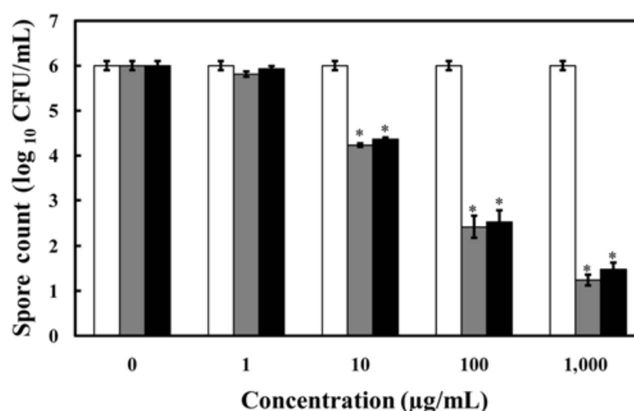

Fig. 3. Sporicidal activity of macelignan and glutaraldehyde at different concentrations for 1 hr on spores of *B. cereus* ATCC 21772. Values are expressed as the average spore count (CFU/mL). *Significantly different ($p<0.05$) from the control (without antimicrobial). □ control; ▨ glutaraldehyde; ■ macelignan.

Hence, killing vegetative cells of a spore-forming bacterium such as *B. cereus* might be easier than that of killing spores of *B. cereus*.

Sporicidal activity Sporicidal activity of macelignan at different concentration (0, 1, 10, 100 and 1,000 μg/mL) against spores of *B. cereus* is shown in Fig. 3. A sharp reduction of *B. cereus* spores density was reached when the spores were exposed to macelignan at a concentration of 100 μg/mL; the reduction in the number of spores/mL was >3 log units (99.9%). Although the complete killing of *B. cereus* spores was not achieved with the treatment by macelignan, the results showed about 99.9% reduction of the spores density with macelignan concentrations as low as 100 μg/mL. Based on this result, 100 μg/mL of macelignan was used to treat the spores of *B. cereus* at different exposure time (0, 0.25, 0.5, 0.75, 1, 2, 3, 4, and 5 hr) (Fig. 4). Even though glutaraldehyde is not well suited for food preservative, glutaraldehyde was reported to have sporicidal activity against spore-forming bacteria (16). There was no significant difference in efficacy between macelignan and glutaraldehyde treatment (Fig. 3 and 4).

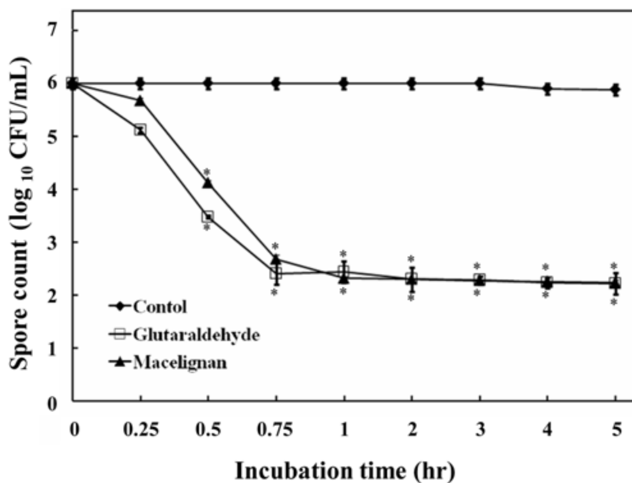

Fig. 4. Sporicidal activity of macelignan and glutaraldehyde at different exposure time on spores of *B. cereus* ATCC 21772. The concentration of macelignan or glutaraldehyde was 100 μg/mL. Values are expressed as the average spore count (CFU/mL). *Significantly different ($p<0.05$) from the control (without antimicrobial).

These results indicate that macelignan has strong sporicidal activity against spores of *B. cereus*.

Report of sporicidal agents isolated from plants is still rare and in reality, simple comparison are difficult because of differences in tested bacteria and theirs concentrations used. Tassou *et al*. (24) reported that oleuropen purified from olive extract inhibited both the germination and the subsequent outgrowth of spores of *B. cereus*. Moreover, a torilin isolated from *Torilis japonica* fruit showed excellent antimicrobial activity against *B. subtilis* vegetative cells and spores. In contrast, lichocalcone A isolated from the roots of licorice (*Glycyrrhiza inflate*), which has various uses in the food and pharmaceutical industries, has antibacterial activity against vegetative cells of *B. subtilis*, but did not inhibit the germination of *B. subtilis* spores (25). In this report, we showed that macelignan isolated from nutmeg exhibited inhibition activity to the growth of vegetative cells and sporicidal activity against spores of *B. cereus*. In summary, it is remarkable to note that macelignan confers significant antibacterial activity against a spore-forming bacterium, *B. cereus*, and sporicidal activity comparable to that of glutaraldehyde. Thus, macelignan might be good to develop as a food preservative. However, the mechanisms of killing require further investigation.

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