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REDUCTION OF AFLATOXIN CONTAMINATION IN NUTMEG COATED WITH CLOVE OIL, PROPYLPARABEN, AND POTASSIUM SORBATE FORMULA

Pengurangan Kontaminasi Aflatoksin dalam Biji Pala yang Disalut Formula Minyak Cengkih, Propil Paraben, dan Kalium Sorbat

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ABSTRACT

Aflatoxin contamination caused by *Aspergillus flavus* in nutmeg has become a severe export obstacle. The study aimed to evaluate the effectiveness of coating formula to reduce *A. flavus* contamination in nutmeg seeds. Cleaned and dried nutmeg seeds were coated with a coating formula containing propylparaben, potassium sorbate, and clove oil, then challenged by spraying with *A. flavus* conidia suspension. The treated seeds were incubated in humid plastic boxes. The colonization of *A. flavus* on the seeds was visually assessed after treatment. Aflatoxin contamination, the residue of propylparaben, and potassium sorbate were analyzed using High-Performance Liquid Chromatography (HPLC). The results showed that total aflatoxin in the shelled nutmeg seeds without coating was 471.69 µg $kg^{-1,}$ which is much higher than that in the coated seeds with formula (4.22 µg kg^{-1}). Also, aflatoxin B1 was 462.10 µg kg^{-1} in the uncoated shelled seeds compared with that in the coated seeds (3.71 µg kg^{-1}). In the unshelled nutmeg seeds without coating, total aflatoxin and aflatoxin B1 contaminations were higher (376.06 µg kg^{-1} and 342.84 µg kg^{-1}, respectively) than that in the coated seeds (3.00 µg kg^{-1} and 2.74 µg kg^{-1}). Propylparaben residue in the coated nutmeg seeds was undetected, while, the potassium sorbate residue was detected as much as 30.86 mg kg^{-1} in shelled and coated nutmeg seeds. The study showed that the coating formula was effective in reducing aflatoxin contamination in shelled and unshelled nutmeg seeds.

[Keywords: Total aflatoxin, aflatoxin B1, *Aspergillus flavus*, *Myristica fragrans*, nutmeg seed coating]

ABSTRAK

Pencemaran aflatoksin yang disebabkan oleh Aspergillus flavus pada buah pala menjadi kendala ekspor yang serius. Penelitian ini bertujuan untuk mengevaluasi efektivitas formula penyalut (coating) dalam mengurangi cemaran A. flavus pada biji pala. Biji pala yang telah dibersihkan dan dikeringkan dilapisi dengan formula penyalut yang mengandung propilparaben, kalium sorbat, dan minyak cengkih, kemudian disemprot dengan suspensi konidia A. flavus. Biji pala yang telah diberi perlakuan kemudian diinkubasi di dalam kotak plastik yang lembap. Kolonisasi A. flavus pada biji pala diamati secara visual. Kontaminasi aflatoksin, residu propilparaben, dan kalium sorbat dianalisis menggunakan High-Performance Liquid Chromatography (HPLC). Hasil penelitian menunjukkan bahwa total aflatoksin pada biji pala yang dikupas tetapi tidak disalut mencapai 471,69 µg $kg^{-1,}$ jauh lebih tinggi dibandingkan dengan biji pala yang disalut (4,22 µg kg^{-1}), sedangkan kandungan aflatoksin B1 dalam biji pala kupas tidak disalut mencapai 462,10 µg kg^{-1}, lebih tinggi daripada dalam biji pala kupas yang disalut (3,71 µg kg^{-1}). Pada biji pala yang tidak dikupas dan tidak disalut, kandungan total aflatoksin dan aflatoksin B1 masing-masing sebesar 376,06 µg kg^{-1} dan 342,84 µg kg^{-1}, lebih tinggi dibandingkan dengan dalam biji pala tidak dikupas tetapi disalut, yaitu masing-masing 3,00 µg kg^{-1} dan 2,74 µg kg^{-1}. Residu propilparaben dalam biji pala yang disalut tidak terdeteksi, namun residu kalium sorbat terdeteksi sebanyak 30,86 mg kg^{-1} dalam biji pala kupas dan disalut. Hasil penelitian menunjukkan bahwa formula penyalut yang mengandung campuran propilparaben, kalium sorbat, dan minyak cengkih dapat mengurangi kontaminasi aflatoksin dalam biji pala kupas dan tidak dikupas.

[Kata kunci: *Aflatoksin total, aflatoksin B1,* Aspergillus flavus*,* Myristica fragrans*, penyalutan biji pala*]

INTRODUCTION

Nutmeg (*Myristica fragrans* Hout) is one of the most important export commodities in Indonesia. In 2015, Indonesia exported 17,027 tons of nutmeg and increased to 19,957 tons in 2016, but the price per ton slightly decreased to USD 4844 (Pakpahan et al. 2020). India was the first world's largest nutmeg exporting country (worth USD 107,906 thousand), followed by Indonesia (USD 96,672 thousand) and Guatemala (USD 95,505 thousand) (Pakpahan et al. 2020). A recent report showed that in 2019, Indonesia produced 37 thousand tons and exported 20 thousand tons for the nutmeg world market, whereas India produced 12 thousand tons and Sri Lanka produced 3 thousand tons (Hafif 2021).

One issue related to the decline of nutmeg export is aflatoxin contamination. Several cases of exported nutmeg from Indonesia to Europe were rejected due to containing aflatoxin, which exceeded the maximum limit. Different countries generally adopt the total aflatoxin B1 and total aflatoxin maximum levels in spices and food products. In Indonesia, the maximum level of aflatoxin B1 is 20 µg kg^{-1}, whereas, in China, India, and Malaysia is 40 µg kg^{-1}, 30 µg kg^{-1}, and 15 µg kg^{-1}, respectively (Benkerroum 2020). A previous study showed that in North Sulawesi, aflatoxin contamination was found at every level of the market chain, from farmers, collectors, to exporters (Dharmaputra et al. 2015).

One critical issue in the management of aflatoxin is the products must be dried and stored below the favorable conditions for *A. flavus* development, i.e., low relative humidity (RH) and temperature (<30°C) (Mannaa and Kim 2018). These conditions, however, were not possible when nutmegs were harvested in the rainy season or no drying facility was available. In this situation, coating nutmeg seed with chemicals might be useful to prevent *A. flavus* colonization.

Methylparaben, propylparaben, and potassium sorbate can inhibit the growth of *Aspergillus* spp. (Mehyar et al. 2012; Bullerman, 1983). Potassium sorbate is widely used in the preservation of food products because it inhibits the growth of many microorganisms, including yeasts, molds, and bacteria (Sofos and Busta 1981). Potassium sorbate at 0.1% had the highest effect on *A. niger* and *Penicillium notatum* Heydaryinia et al. (2011) and at a lower concentration delayed or prevented spore germination and growth initiation, and reduced aflatoxin BI production of *A. parasiticus* and *A. flavus* (Bullerman 1983). Other chemical agents that can be used to inhibit *Aspergillus* growth are essential oils, such as *Trachyspermumammi, Cymbopogon martinii*, and *Foeniculum vulgare* oils (Gemeda et al. 2014). The extracts of *Artemisia dracunculus, Achillea wilhelmsii*, *Bunium persicum, Cuminum cyminum*, and *Zataria multiflora* have antifungal properties against *Aspergillus* sp. (Tajehmiri et al. 2018). The regulatory status of propylparabens in different countries differed. In the United States and Canada, propylparaben can generally be used in food products with a maximum level of 0.1% (Snodin 2015).

During the harvest season of nutmegs in the rainy season or in no drying facility available, nutmeg seeds are susceptible to fungal infection by *Aspergillus* spp. In this situation, coating nutmeg seeds with antifungal substances might be useful to prevent *A. flavus* colonization. The study aimed to evaluate the effectiveness of seed coating formula to reduce *A. flavus* contamination in nutmeg seeds.

MATERIALS AND METHODS

Study Area

The study was conducted in the laboratory of the Indonesian Spices and Medicinal Crops Research Institute from January to December 2019.

Preparing of Unshelled Nutmeg Seeds

Unshelled nutmeg seeds were purchased from a local supplier in Bogor, West Java, Indonesia.

Propagation of *A. flavus* Isolate

An isolate of *A. flavus* producing aflatoxin was obtained from the Microbial Collection Culture of the Southeast Asian Regional Centre for Tropical Biology (SEAMEO BIOTROP), Bogor, West Java (Prof. Dr. Okky Dharmaputra). *A. flavus* isolate was propagated on potato dextrose agar (PDA) medium and incubated at room temperature.

Coating Formula Preparation

Coating preparation followed the method of Acosta et al. with modification (Acosta et al. 2016). The coating formula, i.e. GM (Arabic gum + carboxymethylcellulose) formula, contained active ingredients of 0.8% potassium sorbate (purity 99.02%), 0.1% propylparaben (purity 98.24%), and 1.25% clove oil (eugenol 73.65%), whereas the carriers were 0.5% Arabic gum, 1% carboxymethylcellulose (CMC), 2% hydrolyzed starch, 0.8% glycerin, and 0.1% Tween-80, and distilled water. Arabic gum (2.5 g) was added to 100 ml distilled water in a baker grass, put in a pan containing tap water, and stirred using a glass rod while heating on a stove until gelatinized. The gelatinized Arabic gum was then added with 1 ml of glycerol and 1 ml of 1% Tween-80, then stirred using a food mixer until thoroughly mixed. Ten grams of hydrolyzed starch were gelatinized in 200 ml distilled water, then added with 2 ml of glycerin and 1 ml of 1% Tween-80. Similarly, gelatinized CMC was prepared from 5 g in 200 ml of distilled water, added with 2 ml of glycerol, 3 ml of 1% Tween-80, potassium sorbate solution (4 g 7^{-1} ml distilled water), propylparaben solution (0.5 g 2^{-1} ml DMSO), and 6.25 ml of clove oil emulsified with 1% Tween-80. Finally, gelatinized Arabic gum, starch, and CMC were mixed in a 500 ml capacity of a homogenizer and stirred at ± 10,000 rpm for 3 minutes. The final volume of the formula was 500 ml.

The Casting of Coating Formula for Antifungal Evaluation

The activity of the coating formula was evaluated against a culture of *A. flavus*. The coating formula was cast following the method of Fakhouri et al. (2015) with a slight modification. Ten ml of the coating formula was added into a plastic box (5 cm x 7 cm x 5 cm) and air-dried to form the coating formula sheet. The air dried-sheet of the formula was cut 0.5 cm x 0.5 cm and then placed on the surface of the PDA media that has been inoculated with 100 µl of *A. flavus* conidia suspension three days old. The culture was incubated for two days at room temperature. The experiment was designed in a completely random and replicated thrice. The presence of a clear zone around the pieces of the coating formula sheet indicated the antifungal activity of the coating formula.

Coating of Nutmeg Seeds

Freshly opened unshelled nutmeg seeds were cleaned from debris under tap water. The seeds were then soaked overnight in tap water and NaOH 0.04% in plastic boxes. The use of NaOH followed the method of Kreitschitz and Gorb (2018). The soaked seeds were then cleaned by pressing with a hand palm to remove a slimy layer from the seed surfaces, then dried under the sunlight, before coating.

The shelled nutmeg seeds were obtained by mechanically craking the dried unshelled seeds. The dried unshelled and shelled nutmeg seeds were then dipped in the coating formula (GM) for a few minutes, transferred on trays, and dried in the sun for 1-2 hours or until the coating layers dried as described by Supriadi et al. (2022).

The experiment used a completely random, with three replicates, and each replicate used 30 seeds. As a control, the unshelled and shelled nutmeg seeds were not treated with the GM coating formula. All the treated and untreated seeds were placed in a plastic box lined with moist tissue papers, then inoculated by spraying the conidia suspension of *A. flavus* using a mini perfume sprayer. Parameters observed were the colonization of *A. flavus* on the surface of the seeds, aflatoxin content, and the residue of propylparaben and potassium sorbate.

Analysis of Aflatoxin Contents and Residues of Propylparaben and Potassium Sorbate

The treated and untreated nutmeg seeds were sampled from each replicate and mixed to form one bulked sample. The seeds were then analyzed in a standardized laboratory in Bogor using High-Performance Liquid Chromatography (HPLC) method code 18-5-30/MU/SMM-SIG. The HPLC method followed Bajcic et al. (2021). The protocol code number of the HPLC is 18-5-30/MU/SMM-SIG (the Saraswanti Laboratory). The sensitivity levels of the HPLC for the total aflatoxin and aflatoxin B1 are 0.1623 µg kg^{-1}, whereas for propylparaben and potassium sorbate are 0.92 mg kg^{-1} and 1.06 mg kg^{-1}, respectively.

Data Analysis

Data of the aflatoxins, propylparaben, and potassium sorbate contents in the nutmeg seeds treated and untreated with the coating formula were analyzed using Tukey's test, $P< 0.05$.

RESULTS AND DISCUSSION

Antifungal Activity of the Coating Formula

The coating formula formed a good strength sheet after casting in the square plastic box and inhibited the growth of *A. flavus* on the PDA medium (Figure 1a). The inhibition zone appeared around the cut sheet (Figure 1b).

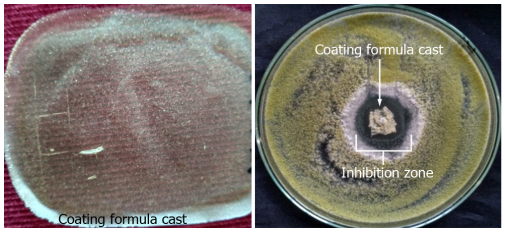
Colonization of *A. flavus* on Shelled and Unshelled Nutmeg Seeds

The untreated shelled nutmeg seeds were heavily colonized with *A. flavus* spores (Figure 2), as seen with a yellowish-green on the seed surfaces, in contrast to those treated with the coating formula that showed no colonization of the fungus (Figure 3). It means that the protective action of the coating on the shelled nutmeg seeds was effective against the colonization of *A. flavus*.

The pre-treatment of unshelled seeds by immersing them in water or NaOH 0.04% solution is essential to improve the seeds' coating performance (Table 1). The coating formula's effectiveness on the unshelled nutmeg seeds was as good as on the shelled ones (Figure 4a), in contrast to those untreated with the coating formula which were heavily colonized with *A. flavus (Figure 4b)*.

Aflatoxin Contamination and Residue of Active Ingredients

The results showed that total aflatoxin in all nutmeg seeds inoculated with *A. flavus* was high. Total aflatoxin of the shelled nutmeg seeds but not coated with the formula was high, i.e., 471.69 µg kg^{-1}, much higher than



a b

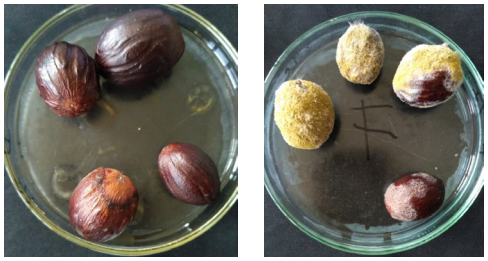
Figure 1. The coating formula sheet (a) and its antifungal activity on *Aspergillus flavus* grown on potato dextrose agar medium (b).

Figure 2. Aspergillus fllavus colonization on shelled nutmeg seeds not coated (left) and coated (right) with Arabic gum + carboxymethylcellulose formula (GM).

Fig 3. A closed-up shelled nutmeg seeds not colonized (left) and colonized (right) with *Aspergillus flavus*.

Table 1. Colonization of *Aspergillus flavus* on the unshelled nutmeg seed surface treated with coating formula.

Treatment	Pre-immersed in water	Pre-immersed in N_aOH 0.04% solution
Coated	7.05	7.31
Control	100.00	100.00



a b

Figure 4. Colonization of *Aspergillus flavus* on the unshelled (a) and uncoated (b) nutmeg seeds.

Table2. The aflatoxin, propylparaben, and potassium sorbate contents in the nutmeg seeds treated with the coating formula*

Treatment	Total aflatoxin (µg kg^{-1})	Aflatoxin B1 (µg kg^{-1})	Propyl-paraben (mg kg^{-1})	Potassium sorbate (mg kg^{-1})
Shelled-not coated	471.69^{a}	462.10^{a}	0.0^{a}	0.0^{a}
Unshelled-not coated	376.06^{a}	342.84^{a}	0.0^{a}	0.0^{a}
Shelled-coated	4.22^{b}	3.71^{b}	0.0^{a}	30.86^{b}
Unshelled-coated	3.00^{b}	2.74^{b}	0.0^{a}	0.0^{a}

Values in the same column followed by the same letters are not significantly different (Tukey's test, P< 0.05).
*Aflatoxins, potassium sorbate, and propylparaben were quantified using high-performance liquid chromatography.

that treated with the coating formula (4.22 µg kg^{-1}). Similarly, on the shelled and not coated seeds, the total aflatoxin B1 was 462.10 µg kg^{-1}, compared with that of the shelled-coated seeds (3.71 µg kg^{-1}) (Table 2). It means that *A. flavus* colonization in nutmeg seeds is very dangerous and could produce high aflatoxin residue. In the unshelled and not coated nutmeg seeds, the total aflatoxin and aflatoxin B1 contaminations were higher (376.06 µg kg^{-1} and 342.84 µg kg^{-1}, respectively) than that in the coated seeds (3.00 µg kg^{-1} and 2.74 µg kg^{-1}). The experiment showed that coating nutmeg seeds with a coating formula were effective in reducing aflatoxin contamination in the seeds. The residue of propylparaben in the coated seeds was below the detection level of 0.92 mg kg^{-1}. However, the residue of potassium sorbate was detected as much as 30.86 mg kg^{-1} only in the shelled nutmeg seeds.

The study offers a coating method to minimize aflatoxin contamination in nutmeg seeds. Aflatoxin contamination is common in spice products, such as black and white peppers, cardamom, coriander, cumin, and nutmeg in Indonesia; therefore, good handling practices are required (Nurtjahja et al. 2019). Aflatoxin contaminations produced by *Aspergillus* spp. in nutmeg seeds are the most severe problem faced by farmers in Indonesia. The problem is caused by limited drying facilities, especially during a rainy harvest season. Protecting the seeds from fungal infections is, therefore, critical.

In the present study, a seed coating formulation has been developed. The formula contains a mixture of potassium sorbate (purity 99.02%), propylparaben (purity 98.24%), and clove oil (eugenol 73.65%). Pre-treatment of unshelled nutmeg seeds in water and 0.4% of KOH solution increased the stickiness of the coating formula to the seed surfaces because the debris and surface layers which were rich in substances to support the growth of fungal colonization, such as *Aspergillus*

spp., have been removed. The present study shows that coating of nutmeg seeds minimizes the total aflatoxin and aflatoxin B1 contaminations. The aflatoxin contents in the shelled and unshelled coated nutmegs are below the maximum limit of 15 µg kg^{-1} for aflatoxin B1 and 20 µg kg^{-1} for the total aflatoxin.

The coating treatments showed a strong correlation for the protection of both shelled and unshelled nutmeg seeds from the colonization of *A. flavus* (r = 1 or 100% correlation), therefore, the coating formula enhanced protection of nutmeg seeds from contamination of aflatoxin B1 and total aflatoxin. The principal antifungal agent in the coating formula developed might be propylparaben. Propylparaben, in combination with potassium sorbate and clove oil, increased the antifungal properties of the coating formula as shown from the bio-assay against *A. flavus*. The coating formula sheet inhibited the growth of *A. flavus* on agar medium.

The same thing occurred when a piece of coated shell was tested against the culture of *A. flavus* in an agar medium (data not shown). Double coatings of unshelled nutmeg seeds might form a thicker layer on the seed surface that can prevent the colonization of *A. flavus* on the nutmeg seed surface. The pH of the coating formula (5.9) is in the range of optimal conditions for propylparaben to act as an antimicrobial agent at a wide pH range (4-8) (Lück and Jager 1997). Propylparaben inhibited the production of *A. flavus* conidia and aflatoxin B1 (Nesci et al. 2003). Also, propylparaben supports the durability of potassium sorbate activity on *A. flavus*.

The residue of potassium sorbate (30.86 mg kg^{-1}) on the shelled-coated seeds needs to be studied to minimize further. Currently, the recommended daily intake of sorbate is 25 mg kg^{-1} (Bajcic et al. 2021). The potassium sorbate intake of more than 25 mg kg^{-1} may lead to health problems such as cytotoxic and genotoxic effects (Dehghan et al. 2018). A study conducted by Siruguri and Bhat (2015) showed that the mean total intake of nutmeg in India is an average of 0.14 g.

The coating method developed in the present study might be used to make other coating formulas using plant's primary or secondary metabolites, such as phenolic, terpenes, and nitrogen-containing compounds, and other substances, such as citrus and curry leaf extracts, and *Annona* seed extract which are effective in reducing *Aspergillus* spp. in pre-and postharvest management of food products (Mathew et al. 2017; Loi et al. 2020). The seed coating application cannot be alone in reducing aflatoxin contamination in nutmeg seeds. The pre-harvest activities, such as only harvesting already mature seeds, indicated with a dark brown color surface and bright red color of mace, and post-harvest activities include drying the seeds as soon as possible, removing broken nutmegs, and other hygienic practices during handing and packaging must be conducted in all market chains before exporting. Simple drying methods, such as drying on the traditional rack stand one meter above soil level and by covering the seeds with a black cover sheet cloth, and drying under the sun have been shown to minimize aflatoxin contamination of nutmeg seeds (Sembiring et al. 2020) a hot-box type equipped with a fan heater, drying on bamboo trays. The unshelled nutmeg seeds were dried until vibrated if shaken and shelled. The temperature, humidity, moisture, oil and aflatoxins contents were analysed. The fiber-house and the electric hot-box types dried the seeds faster (2-3 days. The present study, therefore, can be integrated with the drying standard protocols for good post-harvest practices to minimize aflatoxin problems in the production of nutmeg seeds in Indonesia.

CONCLUSION

The coating formula containing clove oil, propylparaben, and potassium sorbate effectively reduced aflatoxin contamination in nutmeg seeds. The total aflatoxin and aflatoxin B1 in the treated unshelled seeds were 3.0 µg kg^{-1} and 2.74 µg kg^{-1}, respectively; whereas in the coated shelled seeds were 4.22 µg kg^{-1} and 3.71 µg kg^{-1}, respectively. The total aflatoxin and aflatoxin B1 were below the recommended maximum limit (10 µg kg^{-1}). The study suggests that the coating formula could be used to reduce aflatoxin contamination in both shelled and unshelled nutmeg seeds.

CONTRIBUTION

Supriadi and Sri Rahayuningsih are the main contributors, whereas A. Bagem Sembiring, Rusbianto Wijaya, and Dini Florina are members.

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