Coating formula containing clove oil suppresses Aspergillus flavus growth on unshelled nutmeg seeds

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Abstract. Aflatoxin contamination has become a constraint on nutmeg. The study aimed to determine the effectiveness of clove oil coating formula to suppress Aspergillus flavus growth on unshelled nutmeg. Clove oil, Curcuma oil, and coconut shell liquid smoke were evaluated for their fungicidal action on A. flavus and formulated the best one with powder carriers. The seeds were pre-treated with gelatin solution (1%) to improve coating adhesiveness, sprayed with A. flavus conidia, and incubated in humid conditions. Clove oil showed the strongest fungicidal action on A. flavus and was formulated (10%) with MgO, CaSO4, and CaO (1:1:1) powder. The coating formula could inhibit A. flavus growth, but A. flavus still colonized the seed's inner part. The coating formula did not affect the seed's water content, ranging from 9.8-10.22%. Pre-coating the unshelled source with gelatin solution (1%) improved the formula's coverage on the seed surface. The coating was more resistant after mechanical impact by falling from a 30 cm high. The amount of coating formula for treating 1000 unshelled seeds (11.75 kg) was approximately 2.8 kg. The coating formula of clove oil mix with carrier powder minimized A. flavus contamination of nutmeg shells.

Keywords: aflatoxin, coating formula, CaO, CaSO4, MgO

1. Introduction
Nutmeg (Myristica fragrans Hunt) is one of Indonesia’s export commodities from the strategic plantation sub-sector because it generates foreign exchange. Many farmers are involved, thus contributing to the absorption of labor. In 2013, foreign exchange generated from the export of nutmeg was US$ 122,000,000, with the farmers involved amounting to 168,658 households [1]. Indonesia is the largest producer and exporter of nutmeg globally, followed by Grenada, India, Malaysia, Sri Lanka, and Papua New Guinea [2]. Indonesia’s value from the exported nutmeg was around US$96,672 thousand [3].

One of the critical problems in nutmeg export is aflatoxin contamination. Aflatoxin-contaminated nutmeg was found at all levels of the supply chain, such as farmers and collectors, and exporters in North Sulawesi [4]. The highest amount of aflatoxin was detected in nutmeg at the farm level, 141 µg/kg. This amount vastly exceeds the maximum aflatoxins allowed for export to Europe, ten µg/kg [5]. The main contributing factor for aflatoxin contamination in nutmeg is that nutmeg has not applied traditional harvest and post-harvest procedures (SOP). The SOP recommended by Ditjenbun is that the nutmeg is harvested after it is old (the flesh of the fruit is cracked and the surface of the nutmeg shell is dark brown), the moisture content of the nutmeg is <10%, and the whole nutmeg is
separated from the cracked seeds [6]. The handling of aflatoxins must be done early, starting from harvesting the seeds, drying the nutmeg as soon as they are peeled from the meat, sorting the nutmeg's quality and good packaging. Proper drying and storage conditions are critical to prevent grain products from Aspergillus contamination [7].

Old and freshly harvested nutmeg contains a moisture content of about 41.72% [8]. The peeled nutmeg seeds from drying the nutmeg shell contain a moisture content of around 15%. Therefore, drying the nutmeg seeds is a must. However, the nutmeg drying facilities owned by farmers are still minimal, and usually, the nutmeg seeds are dried on a mat and placed directly on the ground. In humid climatic conditions and lots of rain, the nutmeg drying process takes a long time to increase mushrooms' chances. Therefore, it is necessary to process the protection of nutmeg seeds from aflatoxin-producing fungal infections. Several types of plant material (vegetable pesticides) have the Potential to control Aspergillus spp. Various essential oils derived from Trachyspermum Ammi, Cymbopogon martinii, and Foeniculum vulgare could inhibit the production of aflatoxins produced by A. niger and A. flavus [9]. Alcohol extracts from Curcera odollam, cloves, and mahogany (Swietenia macrophylla) could inhibit the growth of Aspergillus [10]. In Indonesia, many types of plants produce essential oils, such as cloves and lemongrass. Therefore, the potential of essential oils to control aflatoxin-producing Aspergillus needs to be investigated.

One technique to protect nutmeg from fungal infection is to coat the seeds' surface with ingredients that inhibit Aspergillus growth. Once recommended, the immersion of nutmeg in quicklime (CaO) solution to prevent rot and pest disturbances [5]. Chalk is known to absorb water and kill mold. The technology of coating the nutmeg with antifungal ingredients has not yet been developed. Besides quicklime, several types of chemical compounds that could absorb water (desiccant) are MgO (magnesia; magnesium oxide) and CaSO4.2H2O (gypsum) [11]. Therefore, the study aimed to determine the effectiveness of a clove oil coating formula to suppress A. flavus growth on unshelled nutmeg seeds.

2. Materials and methods

Researched at the Indonesian Spice and Medicinal Plants Research Institute's disease laboratory in January - December 2018.

2.1. Aspergillus flavus cultivation

The A. flavus fungus isolate used a collection of Biotrop, IPB derived from nutmeg. The A. flavus isolate has been identified to produce aflatoxins (Dr. Okky Dharmaputra, personal communicator). In Petri dishes, A. flavus isolates were propagated on potato dextrose agar (PDA) media and stored as culture collections on slanted PDA media in test tubes.

2.2. Fungicidal screening of clove oil, Curcuma oil, and coconut shell liquid smoke

Clove oil, curcuma xanthorrhiza oil, and coconut shell liquid smoke were evaluated for their effectiveness on A. flavus growth. Benomyl was used as a standard fungicide. The experiment was conducted by mixing the tested materials in a sterilized PDA medium. One ml of the tested material solutions was mixed with 100 ml melted of sterile PDA before pouring into Petri dishes. Five concentrations of the oils, i.e., 100 ppm, 200 ppm, 300 ppm, 400 ppm, and 500ppm, were tested. The PDA media was then inoculated with three-day-old A. flavus cultures. Measured fungal diameter growth after one-week incubation at room temperature.

2.3. Coating formula

The coating formula consisted of clove oil (10%) mixed with MgO, CaSO4, and CaO powder (1:1:1). First, the clove oil (30 ml) was mixed with MgO (100 g), then sieved to get fine powder, then added CaSO4 (100 g) and CaO (100 g) and sieved. The finished coating formula was stored in a plastic bag until used.
2.4. Gelatin adhesive coating solution
Gelatin solutions (0.5% and 1.0%) were tested as a sticky solution to add adhesiveness to the coating formula on the surface of unshelled-nutmeg seeds. The gelatin concentrations were prepared in a liquid smoke solution, as follows:
1. 0.5% gelatin adhesive solution in liquid smoke;
2. 1.0% gelatin adhesive solution in liquid smoke;
3. 0.5% gelatin adhesive solution in liquid smoke + 10% clove coating formula;
4. 1.0% gelatin adhesive solution in liquid smoke + 10% clove coating formula;
5. Water + coating formula without cloves;
6. Water + 10% clove coating formula;
7. Control without treatment.

Unshelled nutmeg seeds were dipped in the adhesive solutions for 5-10 minutes, put in a plastic bag containing a powder coating formula containing clove oil 10%, and shook to cover the surface evenly. Next, the treated seeds were put on the petri dish lids (5 nutmeg seeds per dish), placed in a plastic box lined with wet wipes as a moisturizer, and then sprayed with A. flavus suspension. The treated seeds were incubated for two weeks at room temperature and recorded the growth of A. flavus. The experiment was repeated twice.

2.5. Clove leaf powder and clove oil formula evaluation
The clove powder coating formula consisted of 30 g of clove leaf powder added in 100 g of coating powder. All ingredients were put in a plastic bag and shaken until well blended, then stored in a tightly closed container. The treatment tested were:
1. 2.5% gelatin adhesive and 30% clove powder coating formula + coating powder (100 g);
2. 2.5% gelatin adhesive and 10% clove oil coating formula + coating powder (100 g);
3. Water and clove powder coating formula 30% + coating powder (100 g);
4. Water and clove oil coating formula 10% + coating powder (100 g);
5. 2.5% gelatin adhesive + coating powder (100 g);
6. Water and coating powder;
7. Control without treatment.

Unshelled nutmeg seeds were dipped in a 2.5% gelatin adhesive solution or water for 5-10 minutes, then put in a plastic bag containing 30% clove powder coating formula + coating powder (100 g) or 10% clove oil coating formula + coating powder (100 g). The seeds were shaken as described before to make evenly coat the entire seed surface. The treated seeds were put into the lid of a petri dish (5 seeds per dish) and sprayed with A. flavus suspension as described before. Fungal growth was observed after two weeks of incubation at room temperature.

The experiment was repeated twice using five seeds per replicate. Then, the same experiment was repeated using more seeds (20 unshelled seeds), three replicates. Selected five treated seeds from each replicate, and then the moisture content was measured by the titration method. Colonization of A. flavus was observed visually, and the intensity of the colonization was scored, starting from low (+), moderate (++), and high (+++). Evaluated the presence of A. flavus in the treated seeds’ inner part from 4 seed samples. Broke the shells of the selected source, and a small amount of the peeled seeds were taken and placed on PDA in Petri dishes. After incubation at room temperature for 4-7 days, Recorded A. flavus growth indicated yellowish-green conidia.

2.6. The adhesiveness of coating formula evaluation
Tested the adhesive formula's strength on the coated nutmeg seeds by dipping the unshelled nutmeg seeds into the adhesive formula (10% clove essential oil and MgO powder). Dropped five treated seeds from a 30 cm high. The adhesiveness was evaluated by measuring the weight of the seeds before and after falling.
3. Results and Discussion

3.1. Effect of clove oil and Curcuma oil on the growth of Aspergillus flavus

The effect of clove oil on the suppression of A. flavus growth diameter colony was better than the treatment of Curcuma oil and coconut shell liquid smoke (Table 1). Coconut shell liquid smoke did not appear to affect the growth of A. flavus, while shell liquid smoke seemed to stimulate the growth of A. flavus. Curcuma oil has the effect of suppressing the growth of A. flavus, especially at high concentrations (500 ppm).

### Table 1. Colonies growth of A. flavus on the essential oil, shell liquid smoke, and benomyl treatment.

| Treatment          | Concentration (ppm) | Colony diameter (mm) | Growth suppression (%) |
|--------------------|---------------------|----------------------|------------------------|
| Shell liquid smoke | 100                 | 75.25                | +5.89                  |
|                    | 200                 | 77.33                | +8.91                  |
|                    | 300                 | 74.67                | +5.17                  |
|                    | 400                 | 74.17                | +4.46                  |
|                    | 500                 | 70.33                | -0.96                  |
|                    | 100                 | 55.75                | -21.47                 |
|                    | 200                 | 52.00                | -26.76                 |
| Clove oil          | 300                 | 36.50                | -48.59                 |
|                    | 400                 | 23.83                | -66.43                 |
|                    | 500                 | 10.17                | -85.67                 |
|                    | 100                 | 69.50                | -2.11                  |
|                    | 200                 | 70.25                | -1.05                  |
| Curcuma oil        | 300                 | 60.75                | -14.43                 |
|                    | 400                 | 54.67                | -23.00                 |
|                    | 500                 | 52.75                | -25.00                 |
|                    | 100                 | 0.00                 | -100                   |
|                    | 200                 | 0.00                 | -100                   |
| Benomyl            | 300                 | 0.00                 | -100                   |
|                    | 400                 | 0.00                 | -100                   |
|                    | 500                 | 0.00                 | -100                   |
| Control            | 71.00               | 0                    |

Positive (+) means stimulates growth, and negative (-) means to suppress the growth.

Among the materials tested, benomyl was the most effective at suppressing the growth of A. flavus at all concentrations (100-500 ppm) and showed no increase at all, even at the lowest concentration (100 ppm) (Table 1). Among the two types of essential oil, clove oil is the best compared to Curcuma oil and shell liquid smoke in suppressing fungal growth. At the lowest concentration (100 ppm), clove oil could inhibit 21.47% compared to the control. Conversely, coconut shell liquid smoke at low concentrations (100-400 ppm) stimulated the growth of A. flavus, while at high concentrations (500 ppm), it inhibited the growth of fungi. Therefore, the coating formula is made with clove oil as an active ingredient.

3.2. Effect of gelatin adhesive concentration

The smooth surface of the nutmeg shell makes it difficult for the coating formula to adhere evenly. The gelatin concentration in liquid smoke as an adhesive for a coating formula in flour can improve the quality of the flour formula that sticks to the nutmeg shells' surface. The greater the gelatin concentration, the better the coating formula adheres to the nutmeg shell's surface. The 1% gelatin concentration appeared to be better than the 0.5% concentration. However, due to uneven coverage on the nutmeg shells, after being inoculated by A. flavus, there were still parts of the nutmeg shells colonized by the fungus (Table 2). In addition, the types of gelatin affected the stickiness of the flour formula. The branded gelatin (1%) was better than the local one; that needed more concentration (10%). The local-made gelatin is cheaper and also labeled as “halal” that is needed for food treatment.
Table 2. The effect of gelatin as an adhesive on the quality of the coating layer containing 10% and A. flavus colonization on the surface of nutmeg shells.

| Adhesive and coating treatment | Coating formula condition | A. flavus colonies on the surface of the unshelled nutmeg |
|-------------------------------|---------------------------|--------------------------------------------------------|
| Gelatin 0.5% in shell liquid smoke | The coating layer stick well | The colonization of A. flavus was not evenly distributed on the surface |
| Gelatin 1.0% in shell liquid smoke | The coating layer stick well | The colonization of A. flavus was not evenly distributed on the surface |
| Gelatin 0.5% in shell liquid smoke, clove oil-coated 10% | Thin coating layer, stick well | The colonization of A. flavus was very small and uneven on the surface |
| Gelatin 1.0% in shell liquid smoke, clove oil-coated 10% | The coating layer stick fairly well | The colonization of A. flavus was very small and uneven on the surface |
| Water, uncoated | Nonuniform coating layer | The colonization of A. flavus occurred on half the surface |
| Water, clove oil-coated 10% | Nonuniform coating layer and peeled | The colonization of A. flavus occurred on half the surface |
| Untreated control | - | The colonization of A. flavus occurred on the entire surface |

3.3. The adhesion of gelatin concentration in clove oil coating formula

The gelatin concentration tested was 0.5%; 1%; and 2.5%. For comparison is 1% Latron adhesive. The gelatin solution is mixed with a coating powder carrier of coating powder. Applied the coating formula to the surface of the nutmeg shell, then allowed it to dry. The coating stickiness was tested by mechanical impact from a height of 30 cm, as described before. Repeated test five times. The formula of 2.5% gelatin adhesive in shell liquid smoke shows the best that evenly covered nutmeg shells' surface. On the other hand, the Latron adhesive 1% had uneven coverage, the same as the water distilled as a control treatment (Table 3). Also, the gelatin adhesive formula 2.5% showed stable homogenates during storage.

Table 3. Comparison of the quality of the coating layer on the treatment of clove oil coating formula on nutmeg shell with 2.5% gelatin adhesive.

| No | Adhesive and coating treatment | Coating condition | A. flavus colonies |
|----|-------------------------------|-------------------|-------------------|
| 1. | Gelatin 2.5%, clove oil formula 10%, powder coating (100 g) | Even and a thick coating layer | A. Flavus colonized slightly and evenly (+) |
| 2. | Water, 10% clove oil formula, coating powder (100 g) | Even and thick enough coating layer | A. flavus colonized slightly (+) |
| 3. | Gelatin 2.5%, coating powder (100 g) | Not evenly distributed | A. flavus colonized evenly (+++) |
| 4. | Water and coating powder | Not evenly distributed | A. flavus colonized evenly (+++) |
| 5. | Control (untreated) | - | A. flavus colonized evenly (+++) |

3.4. Effect of gelatin adhesive on adhesion of coating formula

The coating formula's adhesion containing 10% clove oil with coating powder as a carrier material on the nutmeg shell's surface turned out to be quite good after going through a physical impact test by falling the seeds from a height of 30 cm. It means that the coating has relatively firmly adhered to the surface of the nutmeg seeds. The pre-coating with 2.5% gelatin adhesive and shell liquid smoke could adhere the coating formula to the shell seeds' surface better than without the bond. The amount of coating formula that sticks to the nutmeg shell's surface after five times dropping from a height of 30 cm.
cm was approximately 2.8 g/seed, greater than without adhesive (1.43 g / seed). It means that the coating of 1000 nutmeg seeds (± 11.75 kg) needs a 2.8 kg formula.

### 3.5. Clove oil coating

The growth or colonization of *A. flavus* on the control nutmeg's surface without treatment (K +) was visible and had a distinctive yellowish-green color after one incubation. Also obtained the same thing in treating the coating powder, except that the colonization of *A. flavus* was uneven. Colonization of *A. flavus* was more visible in the incidence of nutmeg shells that were not covered with a coating because the coating was unevenly covered. In nutmeg treated with an adhesive formula and a formula containing clove oil, *A. flavus* colonization was only slightly a few days after the inoculation (7 days) (Table 4). Therefore, the coating with clove oil formulas is good enough to protect nutmeg shells against *A. flavus* colonization (Figure 1).

#### Table 4. Condition of coating and *A. flavus* colonization on the nutmeg shells treated with a coating powder and 10% clove oil.

| No | Adhesive and coating treatment | Aspergillus flavus colonies |
|----|--------------------------------|----------------------------|
| 1  | Gelatin 1% in shell liquid smoke, coating powder | The coating covers the entire surface of unshelled nutmeg, the colonization of *A. flavus* was uneven |
| 2  | Gelatin 2.5% in shell liquid smoke, coating powder | The coating covers the entire surface of unshelled nutmeg, the colonization of *A. flavus* was uneven |
| 3  | Gelatin 1% in shell liquid smoke, coating powder, 10% clove oil | Thin coating layer covers evenly, the colonization of *A. flavus* was slight |
| 4  | Gelatin 2.5% in shell liquid smoke, coating powder, 10% clove oil | Thick coating layer covers evenly, the colonization of *A. flavus* was slight |
| 5  | Untreated | *A. flavus* colonized over the entire surface unshelled nutmeg |
| 6  | Coating powder does not contain clove oil | *A. flavus* colonized half on the surface which is not covered with a coating |
| 7  | Clove oil 10% in water | The coating is uneven and peeled, *A. flavus* colonized half on the surface which is not covered with a coating |

![Figure 1](image1.jpg)

**Figure 1.** Colonization of *Aspergillus flavus* on the nutmeg shell's surface without any treated (a), treated with a coating formula containing clove oil 1.0 g (b), 0.5 g (c), and clove powder (d) during two weeks incubation at room temperature.

*A. flavus* is one of the fungi that produce different types of aflatoxin, such as aflatoxin B1 (AFB1), B2 (AFB2), G1 (AFG1), G2 (AFG2), M1 (AFM1), and M2 (AFM2), although not all the species produce aflatoxin [11]. Until now, there is no single factor that could be used to control the fungal contamination in agriculture products, including nutmeg. To minimize *Aspergillus colonization* on nutmeg seeds, the seeds should be dried as soon as possible after being harvested and dried to keep their water content below 10% [12]. Under the rainy season, this practice is difficult, therefore, this
preliminary finding of the effectiveness of coating powder containing clove oil and chemical absorbents (MgO, CuSO4, and CaO) might be useful to minimize Aspergillus contamination on nutmeg seeds. Recently, Sembiring et al. had developed drying facilities models to minimize aflatoxin contamination in nutmeg [13]. Further study is required to analyze the residue of the substances, especially MgO, CuSO4, and CaO in the shelled treated nutmeg. Also, to detect the aflatoxin contamination in the shelled-nutmeg seeds. Aflatoxin contamination in the nutmeg kernel can be analyzed using the HPLC method [14]. Another simple method to detect aflatoxins is indicated by blue luminance on the kernel after exposure to UV light [15].

4. Conclusion
Clove oil has antifungal activity against aflatoxin-producing A. flavus isolates. Therefore, a powder coating formula containing clove oil and coating powders of MgO, CuSO4, CaO (1:1:1) could suppress A. flavus colonization on the nutmeg surface. However, because the nutmeg shell's physical structure was slippery, the coating formula was less sticky. Therefore, immersing the seeds with an adhesive gelatin solution in liquid smoke can increase the formula's adhesion to the nutmeg seeds.

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