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Inhibition of Aflatoxin B1 by Aqueous extract from Green Cardamom

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Abstract

Aflatoxin B1 is a secondary metabolite produced by Aspergillus section Flavi during their development, is one of the most dangerous mycotoxins identified to date, that may contaminate many commodities, especially in tropical and sub-tropical regions. Aflatoxin B1 is believed to be the most essential member of this family of mycotoxins, due to its carcinogenic properties in humans and animals. However, there are many strategies to prevent mycotoxin contamination in which some of them are based on the use of natural compounds that may able to minimize the toxin synthesis pathways. Aims: The current work aimed to identify specific compounds from Green Cardamom that expected to interfere with aflatoxin production pathway. Methodology: The adapted method was based on the incubation of fungal culture with different concentrations of the aqueous extraction of Green Cardamom. Results: The aqueous extract of Green Cardamom was able to inhibit Fungal growth and Aflatoxin B1 production by the toxigenic strain of Aspergillus Flavus in a dose-dependent manner. Discussion: the reduction of Aflatoxin B1 production was significantly higher (94.34%) in comparison with other plant extracts such as Caffeine (50%), and reduced fungal growth with an average of (4%) compared to control. Conclusion: Aqueous Extracts of Green Cardamom show an obvious reduction in inhibition synthesis of Aflatoxin B1 by toxigenic Aspergillus strain, the adapted method was effective, but even further investigation is still recommended.

Keywords: mycotoxins, aflatoxins, inhibition, natural extracts, *Aspergillus flavus*

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Introduction

Aflatoxins are toxic secondary metabolites produced naturally by various species of the Aspergillus genus and in particular by those belonging to the section Flavi. Aspergillus flavus, Aspergillus parasiticus and Aspergillus nomius that classified as a toxigenic species because they are found in numerous crops where they can produce aflatoxins [1,2]. At least 20 different aflatoxin molecules have been identified, four of them being naturally produced by fungi (AFB1, -B2, -G1, and -G2) and the rest corresponding to metabolites that appear

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during liver metabolization of the fungal toxins in mammals [3,4].

Aflatoxin B1 (AFB1) is considered the most important member of this family [36]. This compound is a potent natural carcinogen, classified as a group 1 carcinogenic compound for humans by the International Agency for Research on Cancer [5]. One of the main pathogenic effects is the induction of hepatocellular carcinoma which is a type of cancer with a high mortality rate [37]. This molecule is also teratogenic, genotoxic, and cytotoxic for many types of cells [6,7]. AFB1 is immune-toxic [9], and exposure to this mycotoxin has been closely related to impaired growth in children, including stunting and wasting [10]. AFB1 contamination is a major problem in tropical and subtropical regions where environmental conditions such astemperature and humidity are optimal for fungal growth and toxin production on crops both pre-and post-harvest [38]. In these regions, seeds (peanuts), cereals (maize, rice), and spices are frequently contaminated with aflatoxins [11, 12]. Different studies showed that the concentration of AFM1 was increased with the increase of the sample storage period and the presence of toxic AFM1 in Libya was within the limit of European Regulations [8].

Different strategies are available to reduce AFB1 contamination, ranging from preharvest prevention (good agricultural and manufacturing practices) to post-harvest prevention (adequate storage practices and chemical detoxification) [15]. However, according to worldwide surveys, such measures do not completely eliminate AFB1 contamination [16]. Moreover, once AFB1 is produced, it is very difficult to be degraded or removed from food commodities because it is highly stable, resistant to extreme temperatures, and unaffected by most food processing steps [15,17]. Therefore, It is vital to find a new strategies to limit AFB1 contamination in crops and subsequent the exposure of consumers to this carcinogenic agent. Different alternative strategies are developed to limit AFB1 contamination of foods and feeds. Among them, the use of atoxigenic fungal strains to compete with toxigenic. Recently, different atoxigenic strains or mixtures of strains are now commercially available [18]. The use of natural compounds from plants has also been proposed as a promising alternative strategy to control AFB1 contamination [19]. Numerous studies have shown that extracts of certain herbs, spices, and plants display antifungal and anti aflatoxigenic activity [19–21]. Plants are one of the richest sources of bioactive compounds, which serve not only to communicate between plants and to protect plants against aggressors such as insects, bacteria, and fungi but also to combat various external stresses [23]. Polyphenols, alkaloids, and terpenes are three examples of plant secondary metabolites that hinder the production of AFB1 [22]. Many of these bioactive compounds exhibit a high anti-oxidant capacity that is linked to their primary function in the plant [24].

Cardamom plants grow wild in parts of the monsoon forests of the Western Ghats in southern India. This area has become known as the Cardamom Hills, and until just 200 years ago wild plants from these hills provided most of the world's supply of cardamom which have been traded in India for at least 1000 years [39].

Material and methods:

Solvents and reagents

All reagents including solvents were purchased from VWR International (Fontenay sous bois, France) and were of analytical grade. Mycotoxins standards (aflatoxin B1, B2) were purchased from Sigma (Saint-Quentin Fallavier, France) and dissolved in toluene acetonitrile (98:2) and were stored at -20 °C according to the manufacturer's instructions.

Mycotoxin extraction: Aflatoxins were extracted using chloroform. After filtration on the phase separation filter, extracts were evaporated and dissolved in toluene acetonitrile (98:2) and separated by migration on TLC plates in Ethere methanol water (96:3:1, vol/vol/vol). Toxin concentration was quantified by fluorimetry at 365 nm with limit of quantification of 5 ng/g [40]

Preparation of Green Cardamom Aqueous Extract

A total of 20 g of Green Cardamom was extracted with 1 L of distilled water under mechanical agitation for 15 h at room temperature. The extract was centrifuged for 15 min at 15,000 relative centrifugal field (Sigma Laborzentrifugen Gmbh, Osterode am Harz, Germany). Subsequently, supernatant was filtered through autoclave sterilized Whatman grade 1 filter paper (GE Healthcare Life Sciences, Vélizy-Villacoublay, France). The final sterile extract was stored at +4 °C until use.

40 mg/mL.

Fungal Strain and Culture Conditions.

The Aspergillus flavus strain NRRL 62477 was used for these assays [34]. For all experiments, 1000 spores of A. flavus were inoculated centrally into the culture medium using 10 L of a spore suspension prepared in Tween 80 from a 7-day-old culture (105 spores/mL). The culture medium was composed of 18 mL of malt extract agar (Biokar Diagnostics, Allone, France) and 2 mL of autoclaved Green cardamom extract, prepared at four different concentrations by dilution in water. Control cultures were made by adding 2 mL of water to the initial 18 mL of malt extract agar. Cultures were incubated for 8 days at 27 °C. Each assay was done in triplicate. After incubation, the growth was quantified in terms of the measured colony diameter.

Results and Discussion

Effect of Green Cardamom Aqueous Extract on Fungal Growth

Fungal growth in four different concentrations of Green Cardamom (10, 20, 30, and 40 mg /mL) was compared with the control culture without extract. After 8 days of incubation for both the control and non-control samples, a gradual slight reduction in growth was noticed with the increased concentrations, and the maximum inhibition for the fungus growth was with

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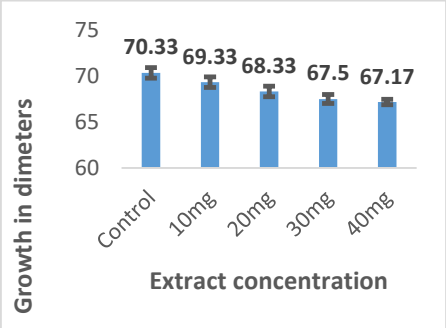

Figure 1: Growth of *A. flavus* in different concentrations of Green Cardamom Aqueous Extract. Results are expressed in mm. Bars on the column show the standard error of the mean (n = 3)

Effect of Green Cardamom Aqueous Extract on AFB1 production

The effect of Cardamom extract concentration on the AFB1 production can be clearly seen in the figure 2. The maximum concentration of AFB1(85.78µg) was obtained by fungal grown on a control culture with no inhibiters been added. Green Cardamom extract showed a dose-dependent reduction in mycotoxin production. The concentration of AFB1 was reduced to 19.28 µg/culture ± 0.60 (reduction of 77.53%) , 12.72 µg/culture ± 0.66 (reduction of 85.17%), 9.32 µg/culture ± 0.57 (reduction of 89.13%) and 4.85 µg/culture ± 0.51 (reduction of 94.34%) when the fungus exposed to 10 , 20, 30 and 40mg /mL of Green Cardamom respectively.

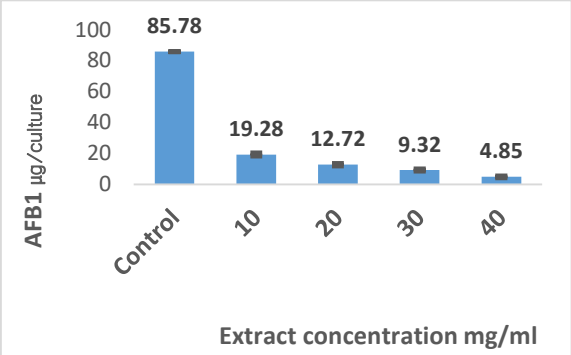

Figure 2: AFB1 production by A. flavus strain NRRL 62477 when exposed to Green Cardamom Aqueous. Results are expressed AFB1 production in µg/culture ± standard error of mean (n = 3).

Conclusion and perspectives

This study clearly demonstrates the effect of Green Cardamom aqueous extract in limiting AFB1 production without altering fungal growth. Such an effect could ensure food safety without affecting biodiversity. This reduction in AFB1 production exceeds that reported for other plant extracts such as caffeine that reduced the toxin production to only 50% and inhibited fungal growth by 91% [35].

However, the slight gradually decreased growth resulted in increased extract concentration suggesting a possible certain concentration could reduce fungal growth to significant levels, which suggested further studies to be performed. Indeed, A. flavus is a very competitive crop-contaminating agent; therefore, the use of uncontrolled fungistatic agents could result in unbalanced environment and favor the emergence of other, possibly uncontrollable microorganisms.

This extract may shelter several bioactive compounds contributing in a complementary way to its anti-aflatoxigenic activity. To ascertain more accurately the inhibitory mechanism of action, the content of this extract needs to be deciphered in order to determine and purify its active molecules as well as the inhibition extent of each of the isolated compounds.

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To continue the work undertaken, several avenues must be explored:

Identification of the active molecule

Although our work has shown that the effect observed on the synthesis of AFB1 was not due to alkaloids such as eugenol, it now remains to identify the active compound(s). For this, a fine separation of the active fractions should make it possible to identify the active compounds responsible for AFB1 inhibition and, then, determine their exact nature.

Effect of aqueous Cardamom extracts on other mycotoxins

Aqueous extracts of Green Cardamom cause inhibition of aflatoxin synthesis, it seems important to characterize their effect on the synthesis of other important mycotoxins such as ochratoxin A and fusariotoxins. Indeed, the biosynthetic pathways of some of these molecules may present similarities with those of AFB1 and thus also be sensitive to the effect of aqueous extracts of Green Cardamom.

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