South Asian Journal of Research in Microbiology

Volume 18, Issue 2, Page 35-41, 2024; Article no.SAJRM.113066
ISSN: 2582-1989

Mycological and Mycotoxicological Producing Potential of Isolates from Fermented Melon Seeds [*Citrullus lanatus* (Thumb) Matsun 'Egusi kirikiri']

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Authors' contributions

This work was carried out in collaboration between both authors. Author CNO designed the study, wrote the protocol and wrote the first draft of the manuscript. Author AA managed the analyses of the study. Author CNO performed the statistical analysis and managed the literature searches. Both authors read and approved the final manuscript.

Article Information

DOI: 10.9734/SAJRM/2024/v18i2347

Open Peer Review History:
This journal follows the Advanced Open Peer Review policy. Identity of the Reviewers, Editor(s) and additional Reviewers, peer review comments, different versions of the manuscript, comments of the editors, etc are available here: https://www.sdiarticle5.com/review-history/113066

Original Research Article

Received: 10/12/2023
Accepted: 14/02/2024
Published: 19/02/2024

ABSTRACT

This study identified and examined fungal presumed producing mycotoxins in fermented melon seed condiment (ogiri). The samples were cultured on Potato Dextrose Agar and Sabouraud Dextrose agar to identify fungi. The detection of mycotoxins in the fermented melon seeds samples was carried out by the use of Coconut Agar Media (CAM), by which the fungal isolates were cultured and plates incubated for 3-7days at 28^{0}C. The mycobiota of fermented melon seed revealed of many species belonging to the *Aspergillus* spp*, Penicillium* spp*, Cladosporium* spp, and *Rhizopus oryzae.* Among all the fungi identified in the study, *Aspergillus* spp has the highest prevalence rate (44.4%) while *Penicillium* spp*,* and *Rhizopus oryzae* have same value (22.2%).the lowest is *Cladosporium* spp with (11.1%) and its presence can be attributed to decaying of the samples. The results obtained show that some of the fungal isolates had the ability to produce

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mycotoxins. From the mycotoxin screening, only six (6) species (*Aspergillus spp, and Penicillium spp)* were positive (+), *Cladosporium spp*, and *Rhizopus oryzae* were negative (-). Mycotoxin contamination level varied in different market locations in which Swali Market had the highest level of mycotoxin (50%) followed by Opolo Market (33.3%) and Tombia Market (16.7%). Mycotoxigenic producing fungi and mycotoxin levels in fermented melon seed samples are public health concern. Meanwhile, the occurrence of the pathogenic fungi (*Aspergillus*, *Penicillium*, and *Rhizopus*) in fermented melon seed can affect its shelf life and can also cause severe health challenges.

Keywords: Food safety; fermented melon seeds; mycology; mycotoxins.

1. INTRODUCTION

"Melon seeds (*Colocynthis citrullus* L. 'Egusi', *Citrullus vulgaris* 'Ahu-elu' and *Citrullus lanatus* (Thumb) Matsun 'Egusi kirikiri') are frequently used as condiment in making soups in Nigeria" [1]. "Melon (*Colocynthis citrullus* L.) is a widely cultivated and consumed oil seed crop in West Africa" [2]. "The seeds, locally called "Egusi" are widely consumed in various forms as a condiment in Nigeria cuisine basically in their local soup" [3]. "Some notable Nigerian delicacies include "Egusi soup". Melon ball snacks and ogiri (a fermented condiment)" [2]. "Ogiri is a Nigerian fermented condiment produced from various substances, and which when added to soup or yam porridge enhances the flavor. Several research has been carried out on the production of *ogiri* from the fermentation of African oil bean seed (*Pentaclethra macrophylla*) creeping melon (*Colocynthis vulgaris*)" [4].

"Fungi are the primary source of mycotoxins, and studies have shown that the incidence of mycotoxin contamination in food is closely associated with the presence of fungi in that commodity" [5]. "Mycotoxins are toxic secondary metabolites produced by fungi that can contaminate a wide range of food including sun-dried meat. Mycotoxins are known to cause a variety of negative health effect to both humans and animals, such as cancer, liver damage, renal failure, and immune suppression, making their presence in food products a critical public health concern" [6]. "Mycotoxin contamination of food resulting from fungal invasion and subsequent biosynthesis of the toxic secondary metabolites is a global challenge, posing a huge hurdle to availability of safe food in regions (e.g. sub-Saharan Africa) where food safety systems are poorly developed" [7] Therefore, this study, focus on major toxigenic fungi and their mycotoxins because identification is an important step towards their control and reduce their economic and health implications.

2. MATERIALS AND METHODOLOGY

2.1 Sample Collection

Tiny melon *Citrullus lanatus* (Thumb) Matsun 'Egusi kirikiri') (*Citrullus lanatus*) seeds were procured from three sites in three markets namely Swalli market, Opolo market, and Tombia market all in Yenagoa Local Government Area, Bayelsa State, Nigeria. A total of three samples, upon collection, the tiny melon seeds were cleaned to eliminate any foreign particles, dirt, or contaminants. The cleaning process involved rinsing the seeds thoroughly under running tap water and air-drying them in a well-ventilated area. Subsequently, the cleaned seeds were soaked in water for 24hrs, grounded and stored in clean, airtight containers to undergo fermentation. These containers were kept away from direct sunlight and maintained at a stable ambient temperature until they were ready for analysis.

2.2 Media Preparation

Potato dextrose agar and Sabouraud Dextrose agar were employed. They were made according to the manufacturer's instructions. "Thirty-nine grams (39g) of dehydrated powder (PDA) and (SDA) were weighed and suspended in 1 litre of distilled water separately, or 1000 ml, in a conical flask. The conical flask was then heated on a hot plate to completely dissolve the agar. The conical flask's mouths were sealed with cotton wool, and the mouths were covered with aluminum foil. The media was then sterilized using an autoclave at 121°C for 15 minutes. After allowing the media to cool, 20 ml was removed and poured into 90 mm sterilized petri dishes, where it would stay for 24 hours to undergo sterilization and solidification" [8].

2.3 Fungal Isolation

The isolation of fungi was carried out according to the agar dilution method as described by [9].

One (1) gram from each sample were homogenized with 90 ml of buffer peptone water and serial dilutions (10^{-1} to 10^{-4}) were performed. Fungal species were isolated on the Potato dextrose agar. The medium was poured into sterile Petri dish and 0.1 ml of each sample suspension was spread-plated onto the PDA media. The plates were incubated for 5 to 7 days at 25°C. Fungal isolates were sub-cultured on Sabouraud Dextrose agar and incubated for 5 to 7 days at 25°C for purification. The total fungal count for each plate was expressed as colony-forming units per gram of sample (CFU/g). Each genus or species identified was then expressed as percentage (%) of the total isolated fungi.

2.4 Identification of Fungi

Fungi isolates were identified and characterized using morphological and microscopic examination as reported by [10]. This was done by observing both microscopic characteristics and morphology of the colonies on PDA and SDA medium.

2.5 Mycotoxigenic Potential of Fungal Isolates

The mycotoxigenic potential of the fungal isolates was determined using Coconut Agar Media (CAM) following a method described by [11] for Aflatoxin (AF), and by [12] for Ochratoxin (OTA), with a slight modification. Coconut Agar (CAM) was used. For the preparation of Coconut Agar Medium, using 300 ml of hot distilled water, a 100 g piece of coconut was homogenized for 5 minutes. After passing through layers of cheesecloth, the homogenate was filtered, and 2 M NaOH was used to bring the filtrate's pH down to 7.0. After adding 20 grams of agar per liter, the mixture was autoclaved for 15 minutes at 120 degrees Celsius to sterilize it. When the media was solid, the pure fungal isolates were cultured on Coconut Agar Media (CAM) and plates incubated for 10 days at 30˚C. When fungal strains grew on Coconut Agar Medium (CAM) they were first screened for the production of Aflatoxin by looking for the emission of blue or green fluorescence at 365 nm following UV light. AF- producing isolates showed green fluorescence on the reverse sides of the plates and a blue-green fluorescence for OTA.

3. RESULTS AND DISCUSSION

The mycobiota of fermented melon seed revealed of many species belonging to the *Aspergillus* spp, *Penicillium, Cladosporium spp*, and *Rhizopus oryzae*. The results obtained include a macroscopic view of fungal colonies recovered from various food commodities (Table 1) using morphological characters such as colony color, colony edge, mycelia color, conidia shape, and microscopic characteristics.

The high colony counts of fungi may be attributed to the environmental humidity this is because, the fermented melon seeds were stored in airtight containers to undergo fermentation, which is in line with the work of [13] who observed that increased environmental humidity increases viability and dissemination of fungi. Table 1 showed that nine isolates were obtained from the fermented melon seed samples used for this study and four pathogenic fungi were identified as *Penicillium sp*p (2), *Aspergillus spp* (4), *Cladosporium spp* (1*)* and *Rhizopus oryzae,* (2). This study is also in line with the work of [14] in which "nine species of pathogenic fungi were isolated from diseased melon (*Colocynthis citrullus* L.) seeds, and some were identified as *Aspergillus niger, Rhizopus oryzae, A. fumigatus, A. flavus, Penicillium* sp, *Curvularia* sp, *Mucor* sp, *Curvularia* sp, *Mucor* sp, *Cladosporium* sp and *Absidia corymbifera"*. Likewise, the report of [15] which state that "*A. flavus, F. solani, R. oryzae, Penicillium* spp., *Mucor* spp., *A. blakelseeana*, and *P. chrysogenum* constituted the natural microflora of Cucurbitaceae seed".

Table 1. Morphological description and identity of fungal isolates

S/N	Sample location	Morphological description	Fungal identity
1	Swali market a	Light green colony with shade-like surface with white edge	*Penicillium sp*p
2	Swali market b	Milk coloured colony with smooth dark surface	*Aspergillus spp*
3	Swali market c	Greenish colony with fluffy surface	*Aspergillus spp*
4	Opolo market a	Milk coloured colony with elevated	*Penicillium spp*
5	Opolo market b	Dark green colony with fluffy surface and white entire edge.	*Aspergillus spp*
6	Opolo market c	White colony with elevated fluffy surface	*Cladosporium spp*
7	Tombia market a	Greenish colony with fluffy white surface	*Aspergillus spp*
8	Tombia market b	White colony with fluffy elevates surface	*Rhizopus oryzae*
9	Tombia market c	White colony with elevated fluffy surface	*Rhizopus oryzae*

Table 2. Frequency of occurrence fungal isolates from fermented Egusi sample

Fungal Species	No of Isolates CFU/g	Percentage (%) of isolation
Penicillium spp	2	22.2
Aspergillus spp,	4	44.4
Cladosporium spp	1	11.1
Rhizopus oryzae,	2	22.2
Total	9	100

Table 3. Mycotoxin preliminary screening of fungi isolated from melon seeds

S/N	Sample location	Probable organism	Mycotoxin-Producing Ability (Blue-Green Fluorescence Intensity Malachova, et al. 2015)	
			AF	OTA
1	Swali market a	*Penicillium spp*	-	+
2	Swali market b	*Aspergillus spp*	+	-
3	Swali market c	*Aspergillus spp*	+	-
4	Opolo market a	*Penicillium spp*	-	+
5	Opolo market b	*Aspergillus spp*	-	+
6	Opolo market c	*Cladosporium spp*	-	-
7	Tombia market a	*Aspergillus spp*	-	+
8	Tombia market b	*Rhizopus oryzae*	-	-
9	Tombia market c	*Rhizopus oryzae*	-	-

Note: AF – aflatoxin; OTA - ochratoxin A

The data revealed that 44.4% of the analysed samples were contaminated with *Aspergillus* species. The result from this work, revealed that *Aspergillus spp* is the prevalent fungi in the samples studied. This can be associated with the deterioration of the samples, which is in line with the work of [14] in which *Aspergillus flavus, Cladosporium spp, A. niger, Penicillium chrysogenum* and *Rhizopus* spp had severe spoilage effects on the inoculated healthy melon seeds. Likewise, it follows the record of [16] in which *ogiri-egusi* and *ogiri-ugba* were analyzed for their fungi and aflatoxin. The research showed that processed ogiri consumed within the sampling area were heavily contaminated and the aflatoxins analysis showed unacceptable levels of aflatoxins. The domination of *Aspergillus* spp is of food safety prime importance because, they are highly toxigenic. The isolation of mycotoxigenic fungi and the predominance of the genus *Aspergillus* in food agree with the work done by [17] and [18]. The genus *Penicillium* was also isolated with 22.2% of the samples contaminated. This survey also revealed the occurrence of *Cladosporium spp*, and *Rhizopus oryzae* in the analysed samples, as shown in Table. 2, the study shows that toxigenic fungi especially *Aspergillus* and *Penicillium* are ubiquitous which may be as a result of the environment where the fermented melon seeds were kept that favored the fungal growth. According to [19], temperature, water activity, and pH influence the growth of fungi in foods. Other factors that controlled the fungal growth include light and nature of substrate [13].

The representative fungal isolated were tested for their ability to produce mycotoxins (AF and OTA), as shown in Table 3. The isolates of *Aspergillus spp* and *Penicillium spp* showed varying intensities of blue and green fluorescence under UV light (365 nm), while the other species did not fluoresce. Although the amounts of AF and OTA were not determined in this study, the chromatography employed in this research revealed the mycotoxins in fermented melon seed samples however, quantification is challenging since this technique is often not sensitive enough for more detail analysis. Therefore, it is used specifically for initial screening and for accurate quantification, more sensitive and advanced techniques will be needed. The presence of these mycotoxigenic fungi has confirmed the potential risk of aflatoxin (AF) and ochratoxin A (OTA) contamination in fermented melon seed. The fluorescence was observed under UV light (365 nm) after 10 days of incubation. While no fluorescence was detected on the non-producer isolates.

Aflatoxins are a group of highly toxic metabolites commonly produced by *Aspergillus* species, such as *Aspergillus flavus*, *Aspergillus parasiticus* [20] and *Aspergillus nomius* [21]. "They can grow on plants in the field, stored foods, and animal

feeds. *Aspergillus* species cause food spoilage and are very common in stored cereals, nuts, herbs, and spices" [22].

Ochratoxins are a group of secondary metabolites with related structures, first discovered in 1965 in South Africa produced by *Aspergillus ochraceus"* [23]. "Several reports have shown that some species in the fungi genera *Aspergillus* and *Penicillium* produce OTAs" [24]. "Species in these different groups are successful contaminants based on climatic conditions. *Penicillium* occupy a wide spectrum of habitats in our environment. As a consequence, many have become economically important in either harmful or useful roles. Some species cause deterioration of wide range of stored products" [25]. *"Penicillium* species are ubiquitous saprophytes that have been identified in the soil, foods, and drinks" [26]. "Some of these *Penicillium* species affect food commodities like cereals, mainly maize and maize products, citrus, pear, vegetables, processed and refrigerated foods like margarine and jam" [27]. "In Cotonou, Benin, isolation of *Penicillium* species have been seen on smoked or dried fishes" [18]. In the same country, [28] confirmed "the presence of *Penicillium* species in millet and sorghum product". "Ochratoxins have been described as group 2 carcinogens" [29]. "OT-producing species in the *Aspergillus* species belong to the *Circumdati* and *Nigri* sections, such as *A. ochraceus* and *A. niger,* respectively" [30]. According to Djossou et al. [31], "the most prevalent *Aspergillus* species in Ivory Coast are *A. fumigatus, A. niger*, and *Aspergillus tubingensis".*

4. CONCLUSION

The research showed the ubiquitous presence of the fungi and mycotoxins in the fermented melon seed samples. The presence of *Aspergillus* spp*, Penicillium* spp, *Cladosporium spp*, and *Rhizopus oryzae* describes the fungal diversity in fermented melon. These fungi, especially *Aspergillus*, the prominent isolated genus in this study, have been reported in various food commodities, indicating that they are significant genus that should not be overlooked. The confirmation in some of the isolates shows that drastic steps must be taken to avert the colossal effects of these contaminants on food quality. Therefore, precautionary measure must be taken to reduce their presence in food.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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