ISSN:
Print - 2277 - 0593
Online - 2315 - 7461

Journal of Natural Science, Engineering and Technology

MYCOFLORA AND AFLATOXIGENIC MOULD ON LEAVES OF *Terminalia catappa*: A COMMON FOOD PACKAGE MATERIAL IN TWO SOUTH-WESTERN CITIES OF NIGERIA

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ABSTRACT

Fungi constitute a major problem in the production, storage and packaging of food. The recent increase in the use of *Terminalia catappa* leaves as a substitute for food packaging materials in Abeokuta and Ibadan necessitated the need to determine the fungal population on the leaves and quantification of aflatoxin produced by them. Fungi species were isolated from leaves of *Terminalia catappa* using standard microbiological procedures .High-Performance Liquid Chromatography (HPLC) was used to quantify the aflatoxin present. Results show toxin-producing fungi and some yeasts inhabit the leaves which include *Aspergillus niger, A. flavus, A. parasiticus, A. niger, Alternaria, Rhizopus*, *Fusarium* and Candida. The fungal count ranged from 1.0×10^6 to 1.9×10^6 (cfu g^{-1}) and 3.0×10^6 to 8.5×10^6 cfu g^{-1} for the surface disinfected and non- surface disinfected leaves respectively from Abeokuta while for Ibadan the fungal count ranged from 1.54×10^6 to 2.0×10^6 and 4.30×10^6 to 12.06×10^6 cfu g^{-1} for surface disinfected and non-surface disinfected leaves respectively. All *Aspergillus* spp. isolated from leaf samples produced aflatoxins G1 and B1 that ranged from 2.64 µg kg^{-1} to 12.50 µg kg^{-1}. There is an urgent need to regulate the use of *Terminalia catappa* leaves as food packaging material in Nigeria because of the presence of toxin-producing organisms it harbours if public health must be protected.

Keywords: Food contamination, Aflatoxin, Terminalia catappa leaves, Packaging materials, Consumers safety.

INTRODUCTION

Packaging materials have been known to be a possible source of microbial contamination of food (Bae *et al*., 2012). Food eaten has a direct influence on health, and packaging materials have direct contact with food. It is therefore important that manufacturers and food handlers keep safe from pathogenic organisms (Hicks, 2003).The earliest food packaging materials were probably leaves from higher plants (Risch, 2009) but over the centuries, food packaging materials have undergone considerable revolution away from natural materials such as leaves to synthetic organic materials such as polyethylene bags(Brody *et al*., 2008). Food marketers frequently target specific consumers with dynamic packaging attributes specifically designed to promote purchase of their food products (Nancarrow *et al*., 1998).

The complexity of food packaging techniques today, reasonably suggests that leaves packaged food items will have little appeal to

consumer taste in modern society (Silayoi and Speere, 2007:, Imran *et al*., 2010). Surprisingly, the use or *Terminalia catappa* leaves in the package of certain products such as cornmeal popularly called *Eko* and corn cake (*Aadun*) in Ogun and Oyo States, Nigeria has enjoyed continued marketing success without changes, and still preferred by consumers.

Terminalia catappa plant is a large tropical tree in the family Combretacea, tolerant of strong wind and salt sprays (Wang *et al.,* 2015). It has traditionally been a very important plant in coastal communities providing a wide range of wood and non-wood products and services.

Hardly is *Terminalia catappa* tree not seen everywhere, in places such as parks, in front of houses in Ogun and Oyo States, Nigeria. Apart from the shield it provides people, the bark of the tree is commonly used as a herbal remedy for malaria and typhoid fever, while the leaves are used as food packaging materials because they are large and wide (Wang, 2015).

Ajala *et al*., (2011) reported the presence of pathogenic bacteria and moulds on leaves such as banana and cocoa leaves as food packaging materials. There is therefore the possibility of the *Terminalia catappa* leaves to contain microorganisms of both bacteria and fungi genera that are harmful and toxin-producing which might affect the food and the consumers of the food. This study, therefore, aims at determining the mycoflora and aflatoxigenic mould that inhabits the *Terminalia catappa* leaves.

MATERIALS AND METHOD

Study Area

Abeokuta and Ibadan are two important cities in south-western Nigeria, located in Ogun and Oyo state respectively. The freely drained, well-aerated sandy soil in these two states makes *Terminalia catappa* plant grow anywhere thus; giving undeniable access to market women that sells food to use as packaging materials without charges.

Sample Collection

The leaves of *Terminalia catappa* were randomly collected from tree of plants from sixty locations in Ogun and Oyo states. Thirty samples were collected from each state, after collection, the samples were labelled and taken to the laboratory in sterile Ziploc bags.

Preparation of Samples Collected

The samples on arrival at the laboratory were divided into two parts, of the thirty samples, collected from Ogun state, were surface disinfected with 2% aqueous solution of sodium hypochlorite for 1minute, then rinsed thrice in sterile water, while the other fifteen were left undisinfected. The same thing was done to the samples from Oyo state. The leaves were aseptically blended in a sterilized blender with peptone water as the filtrate to make a paste.

Isolation and Characterization of Fungi in Terminalia Catappa Leaves (Surface Disinfected and Non- Surface Disinfected).

Enumeration of fungal load from the samples was achieved by pour plate method. Serial dilution of each sample was obtained by pipetting into a test tube containing 9 ml of peptone water, 1 ml was pipetted again from the 10 ml that was made up to another test tube containing 9ml of peptone water and was done in 10 folds. The 10^3-10^6 dilutions were placed on Sabouraud Agar (SDA) at 28 ° C in an incubator for five days and each dilution was made in triplicate, colonies were

counted and expressed in colony-forming unit/gram (CFU/g) of samples. Isolates were identified using morphological and cultural characteristics as highlighted by Klich (2002).

Screening for Aflatoxigenic Potential of Fungal Isolates

Isolates were screened using Bright Greenish-Yellow Fluorescence experiment (BGYF). Strains were inoculated at a central point on a 6 cm diameter Petri dish containing 10 ml of Coconut Agar Medium (CAM) supplemented with 0.3 % β-cyclodextrin for preliminary screening of aflatoxins production (Fente *et al*., 2001) and incubated for 5 days in the dark at 28°C. Culture plates were passed through 365 nm ultraviolent light for fluorescence detection.

Aflatoxin Quantification of Terminalia catappa Leaf Samples

Leaf samples were prepared for analysis and quantified by the method described by Hell *et al*. (2009) without modifications. Ten grammes of each leaf paste were mixed with 50 ml of methanol (BDH Chemicals Ltd Poole, England)/water (85:15 v/v) blended for 3min, and filtered. The filtrate which was about 40 ml was mixed with an equal volume, 40 ml of 10% sodium chloride (BDH Chemicals Ltd Poole, England). The mixture was poured into a separating funnel and defatted with 25 ml of n-hexane (BDH Chemicals Ltd Poole, England). The hexane layer was discarded and the aqueous layer partitioned twice with 25 ml of chloroform (May and Baker Ltd Dagenham, England). The chloroform layers were pooled and dried over anhydrous sodium sulphate (Cambrian Chemicals Beddington, England). The chloroform was then evaporated off on a rotary evaporator and the residue transferred to an amber vial with 0.5 ml of chloroform which was evaporated off under vacuum to near dryness and stored at - 20 °C until analyzed. The residue was re-dissolved in 200µl acetonitrile which was used as the mobile phase at a flow rate of 0.8 ml min^{-1}. The injection volume was 20 µl. The analyses were carried out with aflatoxins standards (Sigma Chemical Company, St. Louis, MO, USA) of known concentrations with AFB1, AFB2, AFG1 and AFG2 eluting at distinct retention time and 0.025µg/ml of each of the standard. The detection limit of the machine with regards to the toxins was 0.001 µg ml^{-1}.

Data Analysis

Data obtained were subjected to statistical analyses using the Statistical Package for Social Sciences (SPSS) version 20.0 (IBM Corp., 2011). Mean values were compared using DMRT. Results were presented as Mean ± Standard deviation. P value less than 0.05 was considered to be statistically significant.

RESULTS

Fungal load on leaves of the non- surface disinfected (NSD) Terminalia catappa from Ibadan ranged from 4.14 × 10^6 cfu g^{-1} to 12.0^6 ×10^6 cfu g^{-1}, a reduction in fungi counts that ranged between 1.50 × 10^6 cfu g^{-1} and 2.0 × 10^6 cfu g^{-1} was recorded for surface disinfected (SD) leaves, while the fungal counts on leaves gotten from Abeokuta for NSD leaves ranged from 3.0 ×10^6 cfu g^{-1} to 8.5 × 10^6 cfu g^{-1} and 1.0 ×10^6 cfu g^{-1} to 1.9 × 10^6 cfu g^{-1} for surface disinfected (SD) leaves (Table 1).

Sixty percent (60%) of fungi isolated from the non- surface disinfected (NSD) and the surface disinfected (SD) leaves from the two locations were from the Aspergillus genera, of most prevent are the *Aspergillus para-*

siticus, . *A. niger, A.fumigatus , A. clavatus, A. nidulans and A. versicolor*. Other fungal genera isolated were Mucor, Rhizopus, Alternaria and Fusarium (Table 2).

Aspergillus flavus, *Aspergillus niger* and *A. fumigatus* were significantly present on the non - surface disinfected (NSD) leaves with values of 97 %, 95 % and 90 % while they occurred on the surface disinfected (SD) leaves at 45 %, 43 % and 43 % , respectively (Fig. 1). Mucor (58 %), Rhizopus(70 %), Alternaria (10 %) and Fusarium (18 %) species also had significant occurrence on the NSD leaves as compared with the SD leaves.

The spores and mycelium of the isolated fungi viewed macroscopically and with microscope presented with varieties of shapes of the spores from oval to single celled and also multinucleated spores. The mycelium were also given off as either septate or aseptate (Table 3).

Most of the mould isolated from the *Terminalia catappa* leaves exhibited strong mycotoxin production potentals. *Aspergillus flavus*, *Aspergillus niger*, *A. parasiticus A. fumigatus* all showed very strong fluorescence indicated by +++ under the ultraviolent light (UV) . *Aspergillus glucans*, *Aspergillus versicolor* and *A. nidilans* showed moderate fluorescence to low (++), while Mucor and Rhizopus did not fluorescence under the UV light (Table 4).

Visual representation of the *Aspergillus* species on petri plates varied from blue- green, yellowish green, brown and black (Table 5).
Aflatoxin quantification from the leaves showed aflatoxin B1 (AFB1) occurring more in the NSD leaves with a minimum value of 3.22 μg kg^{-1} and a maximum value of 12.50 μg kg^{-1} and aflatoxin G1 (AFG1) with a minimum value of 2.64 μg kg^{-1} and 4.50 μg kg^{-1} for its maximum value (Table 6).

DISCUSSION

Mycoflora profile of *Terminalia cattappa* leaves from the two investigated cities compares favourably with the report of Adegunloye *et al*, (2006), who isolated *Aspergillus niger, A. flavus, rhizopus and candida* from leaves of *Musa Paradisica* (banana leaves), a leaf used as food packaging material. The high fungi load isolated from non-disinfected *Terminalia catappa* leaves which comprise mainly of *Aspergillus niger, Aspergillus flavus* and *Aspergillus prasiticus* from the two cities is an indication of serious health issues to individuals that might be used to consuming food from the leaves because of the assertions that the isolated fungi are toxin-producing ones which can affect the food and consequently the food consumers (Omosuli *et al*., 2008). Reduction in the fungi load isolated from the surface disinfected leaves from both cities was due to the effect of the sodium hypochlorite used. This agrees with the study of Ajala *et al*., (2011) that reported significant fungal count on non-disinfected banana leaves which he suggested may be of serious health hazard to individuals that consume the food in them and less on the surface disinfected.

The disinfected leaves from this study showed less fungal count due to the action of the disinfectant used and water. This might be an imperative way to tackle the problems of food poisoning caused by fungi in leaves. This finding is supported by Adegunloye *et al*, (2006) who reported that disinfected leaves used as part of food materials harbour less micro-organisms, increases food shelf life and do not cause a serious

health hazard to the consumer of the food it is packed with.

The abundance of *A. niger, A. fumigatus, A. nidulans, Rhizopus, Fusarium, and Candida*, on both disinfected and non-disinfected *Terminalia catappa* leaves shows that they are conducive habitat for these organisms, especially the *Aspergillus* spp.; they tend to permanently inhabit the leaves probably due to the ability of the trees and leaves' to withstand high temperature and humidity . Their presence could be a possible source of occurrences of food borne intoxication because of their production of aflatoxin (Kabir, 2009).

Nigeria is one of the countries in the tropics faced with food intoxication from so many angles, from cow's milk as reported by Oluwafemi *et al*., (2014) and also from numerous staple foods (Bandyopadhyay *et al*., 2007). Local food packaging materials such as leaves unfortunately from this study have been revealed to harbour mycotoxigenic organisms. Sixty percent of fungi isolated from the leaves from the study are aflatoxin producers, which indicate the possibilities of serious food intoxication challenges. The amount of aflatoxin quantified from the leaves is above the standard regulatory limit of the permissible level of aflatoxin in food. Although there are no limits yet for leaves used as package materials, it is possible that the exact amount of aflatoxin in leaves would be transferred to the food. This study has also shown the need to regulate the use of some leaves as food packaging materials in Nigeria and similar tropical countries, if public health must be protected.

CONCLUSION

The common practice of using the leaves of *Terminalia catappa* in food packaging in Nigeria without proper sanitary cautions should be discouraged ,since aflatoxigenic organism have been ascertained to inhabit the leaves. However further studies need to be conducted on the carry-over of the toxin to the food and the extent of damage it is likely to cause.

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Table 1: Fungal Counts (cfu g^{-1}) for Surface Disinfected and Non-Disinfected Leaves of Terminalia *catappa* from Abeokuta Ogun State and Ibadan, Oyo State

No	Abeokuta		Ibadan	
	Disinfected	Non-Disinfected	Disinfected	Non-Disinfected
1	1.00×10^6	3.00×10^6	1.53×10^6	6.70×10^6
2	1.11×10^6	3.53×10^6	1.56×10^6	4.68×10^6
3	1.34×10^6	4.25×10^6	1.65×10^6	7.82×10^6
4	1.56×10^6	3.46×10^6	1.68×10^6	5.00×10^6
5	1.34×10^6	5.64×10^6	1.50×10^6	10.03×10^6
6	1.34×10^6	8.00×10^6	1.98×10^6	11.09×10^6
7	1.54×10^6	3.71×10^6	1.98×10^6	12.06×10^6
8	1.67×10^6	5.41×10^6	1.67×10^6	7.89×10^6
9	1.90×10^6	7.51×10^6	1.96×10^6	8.95×10^6
10	1.80×10^6	6.53×10^6	1.57×10^6	9.50×10^6
11	1.80×10^6	8.14×10^6	1.54×10^6	10.00×10^6
12	1.90×10^6	4.56×10^6	1.87×10^6	11.00×10^6
13	1.76×10^6	5.67×10^6	1.80×10^6	4.30×10^6
14	1.46×10^6	6.45×10^6	1.60×10^6	4.14×10^6
15	1.54×10^6	7.54×10^6	1.73×10^6	5.87×10^6
16	1.32×10^6	8.29×10^6	1.72×10^6	7.96×10^6
17	1.80×10^6	8.50×10^6	1.80×10^6	8.09×10^6
18	1.42×10^6	4.60×10^6	1.97×10^6	7.98×10^6
19	1.67×10^6	3.79×10^6	1.54×10^6	6.54×10^6
20	1.54×10^6	6.00×10^6	1.99×10^6	7.00×10^6
21	1.70×10^6	6.54×10^6	2.00×10^6	6.98×10^6
22	1.20×10^6	6.53×10^6	1.98×10^6	9.82×10^6
23	1.10×10^6	4.87×10^6	2.00×10^6	8.96×10^6
24	1.56×10^6	5.00×10^6	1.67×10^6	7.67×10^6
25	1.43×10^6	4.89×10^6	1.89×10^6	8.90×10^6
26	1.57×10^6	4.70×10^6	1.90×10^6	10.50×10^6
27	1.65×10^6	3.90×10^6	1.60×10^6	6.70×10^6
28	1.78×10^6	4.78×10^6	1.49×10^6	5.90×10^6
29	1.60×10^6	4.00×10^6	1.50×10^6	9.08×10^6
30	1.30×10^6	7.00×10^6	1.87×10^6	8.09×10^6

Table 2: Fungi Isolated from Non-Disinfected (N.D) and Surface-Disinfected (S.D) Leaves of *Terminalia catappa*[a]

Fungal species	Non-disinfected leaves Abundance (%)	Disinfected Leaves Abundance (%)
Aspergillus parasiticus	33[a]	18[a]
Aspergillus niger	95 [a]	43[b]
Aspergillus fumigatus	90 [a]	43[b]
Aspergillus flavus	97 [a]	45[b]
Aspergillus clavatus	12 [a]	7 [a]
Aspergillus nidulas	8 [a]	5 [a]
Aspergillus versicolor	3 [a]	2 [a]
Aspergillus glucaus	27 [a]	15 [a]
Alternaria species	10 [a]	0[b]
Mucor species	58 [a]	22[b]
Rhizopus species	70 [a]	25[b]
Fusarium species	18 [a]	12[a]

[(a,b)] Opposite superscripts along rows are significantly different.

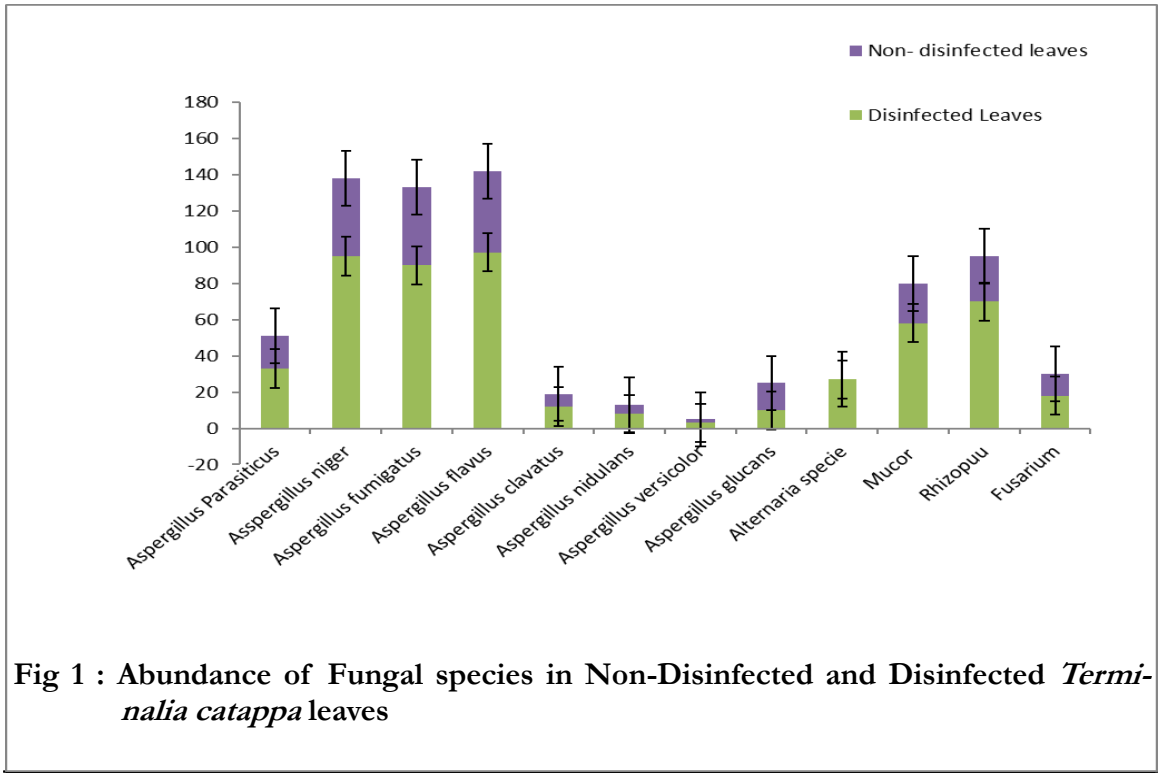

Fig 1 : Abundance of Fungal species in Non-Disinfected and Disinfected *Terminalia catappa* leaves

Table 3: **Cultural and Morphological Characterization of Fungal Isolated**

Isolates	Features		Probable species
	Microscopic	Macroscopic	
F1	Spores are oval; non septate brown mycelium give rise to straight sporangiophore that terminate with black sporangium containing a columella; with rootlike hyphae (rihizpoids)	Rapidly growing white coloured fungus swarms over entire plates; aerial mycelium cottony and fuzzy	Rhizopus species
F2	Spores are oval; non septate mycelium gives rise to single sporangiphores with globular sporangium containing a columella; there are no rhizoids	Rapidly growing white coloured fungus swarms entire plates	Mucor species
F3	Multicelled spores (conidia) are pear-shaped and attached to single conidioophores arising from septate mycelium	Grayish-black colonies with grey edges rapidly swarming over entrie plate; aerial myceloium not very dense, appears Grayish to white	Alternaria species
F4	Multicelled spores (conidia) are oval and attached to conidiophore arising from a septate mycelium	Wolly, white and funzzy colonies changing colour to pink	Fusarium species
F5	Single-celled spores (conidia) in chains developing at the end of sterigma arising from the terminal bulb of the conidiophore, the vesicle; long conidiophore arise from a septate mycelium	White colonies becomes black, blue-green, yellow-green etc.	Aspergillus species

Table 4 : Aflatoxigenic Potentials Identified in Fungal Isolates

S/N	Fungal isolates	Aflatoxigenic Properties
1	*Aspergillus fumigatus*	+++
2	*Aspergillus parasiticus*	+++
3	*Aspergillus flavus*	+++
4	*Mucor* spp.	-
5	*Rhizopus* spp.	-
6	*Aspergillus niger*	+++

+++ Strong fluorescence ++ Moderate fluorescence - No fluorescence

Table 5: Colour of *Aspergillus* Species on the Surface and Reverse Side of the Agar Plate

Species	Surface	Reverse
A. clavatus	Blue-green	White, brownish with age
A. flavus	Yellow-green	Goldish to red-brown
A. fumigates	Green with yellow areas	White to tan
A. glaucus group	Green, to yellow	Yellowish to brown
A. nidulans	Green, to yellow	Purplish red to olive
A. niger	Black	White to brown
A. terrus	Cinnamon to brown	White to brown
A. versicolor	White at the beginning turns to yellow	White to yellow

Table 6: Concentrations of Aflatoxin B1 and G1 quantified from Leaves of *Terminalia catappa*

Sample No	AFG1(µg/kg) (NDL)	AFB1(µg/kg) (SDL)	AFG1(µg/kg) (NDL)	AFB1(µg/kg) (SDL)
1	4.50	12.50	1.50	10.30
2	2.64	3.29	1.34	1.55
3	2.27	3.22	1.10	2.08
4	3.55	7.36	0.50	4.67
5	2.95	5.90	0.78	3.22
6	2.37	3.26	0.67	1.09
7	4.10	6.93	1.57	4.09
8	4.02	4.56	1.40	2.39
9	3.20	5.45	0.00	1.55
10	2.98	5.46	0.01	3.87

AFG1: Aflatoxin G1, **AFB1**: Aflatoxin B1, **NDL** : Non- Disinfected Leaves, **SDL**: Surface- Disinfected Leave

(*Manuscript received: 16th April, 2021; accepted: 30th August, 2021)*