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Microbial quality and proximate composition of dried *Hibiscus sabdariffa* calyxes in Uyo, Eastern Nigeria

Adebayo-tayo, B. C.* and Samuel, U. A

Department of Microbiology, University of Uyo, Uyo Akwa Ibom State, Nigeria

E-mail: bukola_tayo@yahoo.com

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ABSTRACT

Dried *Hibiscus sabdarifa* calyxes collected from different markets in Uyo, Eastern Nigeria were evaluated for microbial quality, Aflatoxin contamination and proximate composition. The results showed that all the calyxes were contaminated with microorganism. The total bacteria count ranged from 5.0 x 10^3 to 8.1 x 10^4 cfu/g in which the highest count was obtained from dried calyxes from Itam Market. Coliform were not detected in most of the sample except samples from Uyo main market and Ikot Ekpene market in which the coliform level is below the acceptable limit. *Salmonella*/*Shigella* was not detected in the sample. The fungi count ranged from 3.4 x 10^4 to 7.3 x 10^4. The associated bacteria were *Staphylococcus aureus, Bacillus subtilis, Bacillus* sp*. Enterococuss faecalis, Micrococcus* sp. and *Klebsiella* sp. The associated fungi were *Aspergillus flavus, A. terreus, A. glaucus, Penicillium citrinum, Fusarium oxysporum, Rhizopus* sp*.* and *Mucor* sp. *A. glaucus* had the highest frequency of occurrence among the isolated fungi. Out of the sample obtained Aflatoxin B_1 was detected in two samples and it ranged from 1.57 to 17.8 /µg/kg. The proximate analysis revealed that the crude protein ranged from 8.34 – 9.97%, crude fibre (7.26 – 7.82%) and fat (8.51 – 9.26%). The moisture content ranged from 13.13 – 14.85%.

Keywords: Hibiscus sabdariffa, calyxes, microbial quality, Aflatoxin, *Aspergillus glaucus.*

INTRODUCTION

Roselle (*Hibiscus sabdariffa*) an herbaceous upright plant species from the family *Malvaceae* is widely grown in the North Eastern and middle belt regions of Nigeria (Akanya *et al.,* 1997). It is locally called "Zoborodo" (Hausa), "Isapa" (Yoruba) and Sorrel in English. Many parts of Roselle are of value, in countries like India, roselle calyxes are utilized in producing refreshing beverages, jellies, jam, sauces and food preserves. (Cyldescale *et al.,* 1979). In Nigeria, the dried roselle calyxes are prepared into a nutritious refreshing drink called 'zobo'. The drink is becoming popular because it is easily prepared at home and served chilled, packed in plastic bottles or polyethylene films. It serves as income generation source for many women.

However, the dried calyxes like any other raw material or food is susceptible to deterioration by food borne microbes which can lead to reduction in quality of the drink in terms of color, taste and nutrition. Most of the fungal contaminant can cause spoilage and they are known to produce mycotoxins which are detrimental to human health. Aflatoxin, a group of toxic metabolites produced by certain *Aspergillus* species have been found to be carcinogenic, tetragenic and mutagenic to several species of experimental animals (Butler and Barnes 1998, Gopalan *et al.*, 1972 and Adamson *et al*., 1973). Now despite the consumer acceptability of drinks from *H. sabdariffa* calyxes and physiochemical evaluation of the "zobo", (Amusa *et al*., 2005, Osuntogun and Aboaba 2004) inhibition of fungal isolates from sorrel drinks (Ilondu and Iloh 2007), fermentation studies on Roselle (Ojokoh *et al*., 2002), research has not been done on the microorganism that causes *H. sabdariffa* calyxes deterioration during storage.

The aim of this study was to investigate, detect and evaluate the presence of microorganism, aflatoxin and proximate composition of *H. sabdariffa* calyxes marketed in Akwa Ibom State, Eastern Nigeria with special references to its public health hazard.

MATERIALS AND METHODS

Collection of Samples

Hibiscus sabdariffa calyxes (Red variety) were obtained from different spot in a local market in Uyo (Akpan Andem Market, Uyo main market, and Itam market) Akwa Ibom State, Nigeria.

One hundred grams of the calyxes were thoroughly washed in distilled water to remove sand and extraneous materials. 10 g of the washed calyxes was weighed into 90 mL sterile 0.1% peptone water as described in the Bacteriological Analytical Manual (FDA, 1991). Ten fold dilutions of each of the samples were made and 0.1 mL of the diluents were pour plated in triplicate plates on Nutrient agar for total bacteria counts, MacConkey agar (Oxoid) for coliform count, Salmonella/Shigella agar for

***Corresponding author**

Salmonella/Shigella counts and Sabourad dextrose agar with chloramphenicol (250 mg/100 mL) for fungal counts. All plates were incubated for 48 h at 30 °C except Sabouraud Dextrose agar that were incubated at 26 °C for 6 days.

Colonies were selected randomly, bacteria cultures were characterized and identified using various morphological and biological test such as Gram stain, spore stain, motility, catalase, coagulase, indole, MR –VP, urease, citrate, oxidase and sugar fermentation. Pure cultures of each isolate were obtained by streaking the specific colonies on suitable media and incubated appropriately; these were maintained in an agar slant in McCartney bottles.

The identification of the microbial isolates was based on classification Scheme proposed by Harrigan and McCance (1976), Buchanan and Gibbson (1974) and Collin and Lyne (1984). The identification was based essentially on morphological and biochemical reactions. The associated fungi were then identified with reference to Barnet and Hunter (1972) and Frazier and Westholf (1998).

The proximate composition was carried out according to the method of Association of Official Analytical Chemists (1990). This includes determination of pH, moisture content, ash content, crude fat, fiber, fat and crude protein and carbohydrate was determined by difference. The nutritionally essential elements (Na, K, Ca and P) were determined using Atomic Absorption Spectrophotometer (AAS).

Detection of aflatoxin in *H. sabdariffa* calyxes' samples

Aflatoxins were extracted from the calyxes according to the method of Seitz and Mohr (1977). Ten grams of the calyxes sample obtained from each of the markets were extracted with chloroform and concentrated.

Thin layer chromatography of aflatoxin and samples extracted were performed on silica gel DG 254 of the extracted sample in which 5, 10, and 15 mL were spotted on three different points on a ruled base line of the TLC plates. Also 5, 10 and 15 mL of the aflatoxin standard were spotted on another three points near the previous sample extract spotted points. The plates were developed first with diethyl ether and then with chloroform acetone (9:1 v/v). Aflatoxins were identified on the basis of co-migration with aflatoxin standards (Fluka) and by their characteristic fluorescent color under long ultraviolet (UV) illumination at 360 nm. The fluorescent spots of aflatoxins were scraped off the TLC and eluted by chloroform methanol (9:1 v/v). The solvent was evaporated under nitrogen to dryness and the residue was dissolved in methanol. The concentration of aflatoxins B_1 in solution was determined by measuring its absorbance at 360 nm then calculated according to the method of Masri *et al*. (1969).

Confirmatory test for aflatoxin

Three different derivatives were prepared by treating portion of the isolated toxin on the aflatoxin standard with formic acid thionyl chloride, acetic-thionyl chloride and trifloroacetic acid. The test was then continuing according to the method of Stolloff (1976).

RESULTS AND DISCUSSION

The total plate counts, coliform count, *Salmonella/Shigella* count and fungi associated with dried *H. sabdariffa* calyxes are shown in Table 1. The result showed that virtually all the calyxes' samples did not have coliform and *Salmonella*/*Shigella* excepts samples from Uyo market and Ikot Ekpene market in which the coliform count was below acceptable limit (10^2 or WHO (1989) standard for food samples).

The total bacteria count ranged from 5.0×10^3 –1.1×10^4 cfu/g in which samples form Ikot Ekpene market had the highest. The fungal count ranged from 3.6×10^4 – 7.3×10^4 cfu/g.

Table 1: Total count of microbial groups in the *H. sabdariffa* calyxes (cfu/g)

Source	Sample	Total bacteria count (10^3)	Coliform count (cfu/g)	*Salmonella*/*Shigella* count (cfu/g)	Fungal count (10^4)
Itam market	A_1	8.1	N.G	N.G	3.6
	B_1	6.4	N.G	N.G	3.4
Akpan idem	A_2	5.5	N.G	N.G	4.3
market	B_2	5.9	N.G	N.G	6.1
Uyo main	A_3	5.0	$\angle 10^2$	N.G	5.4
market	B_3	7.9	N.G	N.G	4.9
Ikot Ekpene	A_4	13.0	$\angle 10^2$	N.G	5.8
market	B_4	11.0	N.G	N.G	7.3

N.G – no growth

Table 2: Microorganism associated with *Hibiscus sabdariffa* calyxes in Akwa Ibom State

Location	Sample	*S. aureus*	*Enterococus faecalis*	*Micrococcus* sp.	*B. subtilis*	*Bacillus* sp.	*Klebsiela* sp.	*Aspergillus flavus*	*A. ferreus*	*A - glaucus*	*Penicillum expamsum*	*Fusarium*	*Cladosporium* sp.	*Candida* sp.	*Saccharomyces* sp.	*A. niger*	*Mucor* sp.	*Streptococcus* sp.
Itam Market	A1	-	-	-	-	-	-	-	+	+	+	-	+	-	+	+	+	+
	B1	-	-	+	-	+	-	-	-	+	+	-	-	-	-	-	-	-
Akpan Idem Market	A2	+	+	-	+	-	-	+	+	+	-	+	-	-	-	-	-	+
	B2	+	-	-	-	+	+	+	-	+	+	+	-	-	+	+	+	-
Uyo main Market	A3	+	+	-	+	+	-	+	+	+	-	+	+	+	-	-	-	-
	B3	-	+	+	-	-	+	+	-	+	-	+	+	+	+	-	-	-
Ikot Ekpene Market	A4	+	-	-	-	+	-	-	+	+	-	-	-	-	-	+	-	-
	B4	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-

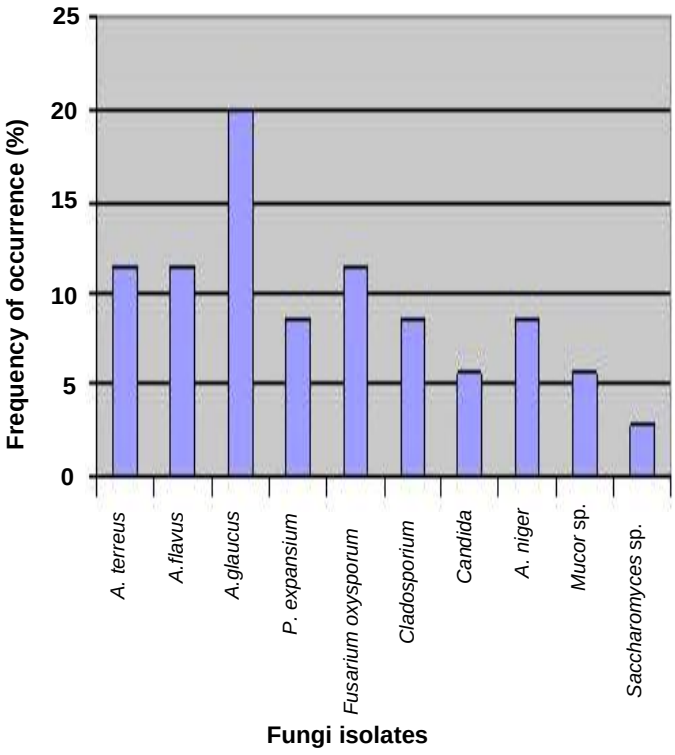

Figure 1: Frequency of occurrence (%) of the fungi isolated from dried *H. sabdariffa* calyxes

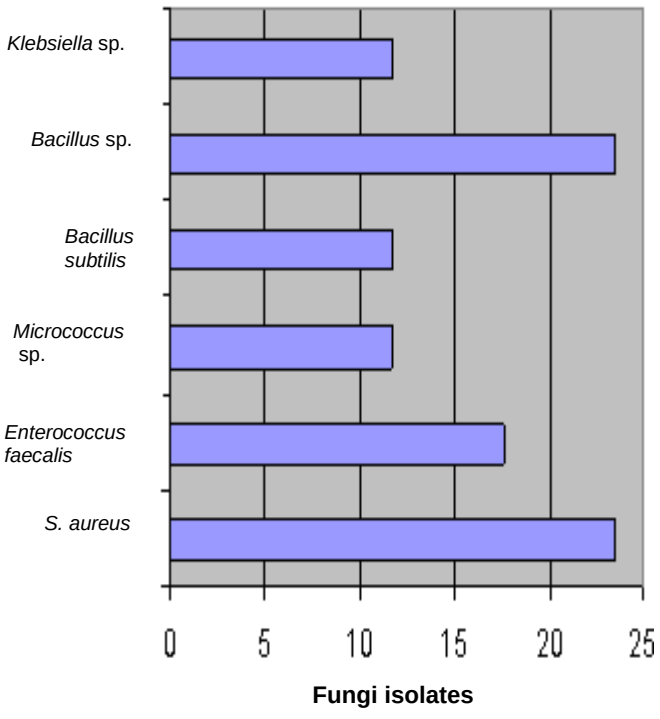

Figure 2: Frequency of occurrence (%) of the bacteria isolated from dried *H. sabdariffa* calyxes

Table 3: Aflatoxin B_1 Content in dried *H. sabdariffa* calyxes obtained from four different markets in Akwa Ibom State

Sample	Itam market	Akpan Idem market	Uyo main market	Ikot Ekpene Market
	B_1	B_1	B_1	B_1
A_1	-	11.67	-	-
B_1	-	-	+ve	0.0
A_2	-	-	15.76	-
B_2	-	-	-	-
A_3	-	15.76	-	-
B_3	-	0.00	-	-
A_4	-	0.00	-	-
B_4	-	0.00	-	-

+ve = detected
- = not detected

The microorganism associated with dried *H. sabdariffa* calyxes are shown in Table 2. The associated bacterial *were Staphylococcus aureus, Bacillus subtilis, Bacillus* sp., *Enterococcus faecalis, Micrococcus* sp., *Klebsiella* sp*.* The fungi and bacteria associated with the dried *H. sabdariffa* calyxes were shown in Figure 1 and 2 respectively. The fungi isolates were *Aspergilles flavus, A. terreus, A. glaucus, P. expansium, Fusarium oxysporum* and *Cladosporum.* Of the six fungi species, *A. glaucus* were the dominant species. *Staphylococuss* and *Micrococcus* species were possible contaminants from handlers. *Staphylococcus aureus*, a mesophile have been implicated in food poisoning outbreak of some food material. Odunfa (1988) reported that *Staphylococcus aureus* levels of 10^8 mL were considered potential hazardous to consumers. The presence of *Staphylococcus aureus* is an indication of contamination by food handlers. 80% of them are being harbored by man as normal micro flora.

The fungi found associated with dried *H. sabdariffa* calyxes were mainly species of *Aspergillus.* This could be attributed to the prevalence of their spores in the atmosphere. The fungi species was easily trapped during the post harvest processing and handling of *H. sabdariffa* calyxes. Since most fungal spores are found in the air, the spores must have contaminated the calyx during drying. The liberated spore can easily settle on food and ceilings of room and then germinated (Okhuoya and Ayanlola, 1986).

Dongo and Ayodele (1997) have shown that *Aspergillus* occurred highest in the number of colonies identified from air spora of some localities. Ilondu and Iloh (2007) isolated and identified *A. flavus* and *A. niger* from sorrel drink.

In the open market, *H. sabdariffa* calyxes are displayed in large bowls and polyethylene bags for prospective consumers and in the process exposed to microbial contamination. Most of the microorganisms isolated have health implications for man except *Micrococcus* sp*.* which have not been associated with human infections. It has occasionally been isolated from human clinical specimen where it mostly represents contaminants from the skin or mucous membrane surfaces or from the environment (Koneman *et al*., 1992).

The occurrences of *Bacillus* sp*.* could be as a result of prevalence of their spores in environment (Jay, 1978). *Bacillus sp*ecies formed spore which could survive high temperatures of processing. *Bacillus* have been isolated from non-alcoholic beverages (Osuntogun and Aboaba, 2004, Amusa *et al.,* 2005). Occurrences of *Enterococcus faecalis* during this work may be as a result of bad habit of the handlers of the dried *H. sabdariffa* calyxes, such as sneezing and coughing without covering their mouth. (Hobbs and Gilbert, 1978).

The concentration of aflatixin B_1 in the dried calyxes is shown in Table 3. It ranged from 0.00 – 15.76 µg/kg. Aflatoxin B_1 was detected in samples from Uyo main market and Akpan idem market. Aflatoxin B_1, secondary fungal metabolites has been reported to be responsible for several ailments in animals including man (Fennel *et al.,* 1973). This aflatoxin is highly carcinogenic causing hepatoxin and has been associated with acute hepatitis in men (Eaton and Groupman 1994). Prolong intake of these contaminated materials can therefore constitute to health risk and significantly reduce net population growth rate. Different species of *Aspergillus* and toxigenic fungi has been isolated from "zobo" juice and sorrel drink ("Zobo") (Omemu *et al*., 2006; Ilondu and Iloh, 2007). Incidence of aflatoxin in dried calyx's samples can be reduced by proper handling methods, adequate storage facilities and planting of resistant varieties.

Table 4: Proximate composition of dried *H. sabdariffa* calyxes (%)

	Moisture content	%dry matter	Ash content	Crude protein	Crude fiber	Fat content	Total carbohydrate
A_1	14.85	85.12	5.92	8.34	7.82	9.26	68.66
B_1	14.61	85.39	6.03	8.65	7.69	9.15	68.53
A_1	13.48	86.52	6.54	9.27	7.26	8.53	68.40
B_2	14.59	85.41	6.05	8.57	7.71	9.18	68.49
A_3	13.45	86.55	6.57	9.36	7.28	8.51	68.28
B_3	13.17	86.83	6.74	9.86	7.43	8.97	67.00
A_4	13.13	86.87	6.77	9.97	7.47	8.95	66.87
B_4	13.28	86.72	6.62	9.64	7.31	8.63	68.13

From the result of this study, it has been made clear those contaminants and toxigenic fungi isolated from sorrel drink was as a result of contamination of the dried calyxes of *H. sabdariffa*.

The implication of this report is that most of the dried *H. sabdariffa* calyxes presently on sale in our markets are not totally acceptable for production of "Zobo" for human consumption. Though the calyxes are properly boiled for the production of "Zobo", once the raw materials are contaminated with aflatoxin boiling does not have any effect on the potency of the toxic materials. Aflatoxin has been found to be heat stable with a melting point of between 268 to 269 °C (Frazier and Westhoff 1998).

Nutritional analyses of the dried *H. sabdariffa* calyxes' are shown in Table 4. It was observed that the protein content, crude, fat content and carbohydrate content were very high. Roselle is often mentioned as an energy candidate yielding fiber, beverage, edible foliage and an oil seed. The extract of Roselle ("Zobo" drink) has been recommended for lowering blood pressure (Varro *et al.*, 1981).

In conclusion, it is therefore important that both the grower and marketer of *H. sabdariffa* take necessary precautions in preventing contamination of the calyxes with fungi to reduce possible contamination and hence reduce the risk of aflatoxin and other mycotoxins that are deleterious to human health. Since the fresh calyxes is eaten raw in salads, cooked and as flavoring in cakes, wine making, beverages, syrup gelatin, puddings and dried calyxes is used for tea, jelly marmalade, ice-cream, butter, pies, sauces, soup tart (Duke and Atchley, 1984; Facciola, 1990) and zobo.

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