Identification and characterization of fungi in *hibiscus sabdariffa* (zobo) drink produced and hawked in eket metropolis, nigeria

Abstract

Zobo drink is an affordable local soft beverage derived from the calyx of *Hibiscus sabdariffa*, a herbaceous medicinal plant that thrives in tropical regions. The characterization and identification of fungi present in Zobo drinks produced and sold by vendors in Eket, Akwa Ibom State, was the aim of this study. Hawked Zobo drinks packaged in plastic bottles were purchased from ten different hawkers, two each from five locations. The pH values of the samples were determined and the total fungal count. The pour plate technique, using Sabouraud Dextrose Agar (SDA) was used to determine the total fungal count. Macroscopic and microscopic features were used to characterize the isolates obtained from the samples. The microscopic identification was done by preparing a wet mount of isolated colonies and observing under a microscope. The rate of growth, colony colour, colour changes, colour of reverse colonies, odour and texture of surface were observed and compared with previous work done on fungi. Results showed that the pH of the freshly obtained and stored samples was 3.9 and 2.8 respectively. The average number of colonies for samples stored in the refrigerator ranged from 0.54×10^5 to 0.91×10^5 cfu/ml while that of the freshly obtained samples ranged from 1.26×10^5 to 1.64×10^5 cfu/ml at 10^{-3} dilution. A total of 43.6% *Aspergillus spp.,* 29.5% *Candida spp.* 15.4% *Penicillium spp.* and 11.5% *Rhizopus spp*. were identified. With increased acidity in the refrigerated samples, the number reduced to 41.2% *Aspergillus spp.,* 27.4% *Candida spp.* 15.7% *Penicillium spp.* and 15.7% *Rhizopus spp*. The fungi species isolated are known to produce mycotoxins with potential immunosuppressive and cytotoxic effect. These microorganisms could have been introduced to the drinks at various stages of production, during transportation, through the ingredients utilized in the manufacturing process, or even during the cultivation of the *Hibiscus sabdariffa* plant. This issue can be mitigated by implementing effective manufacturing practices throughout the production process.

Keywords: Zobo; Fungi; Characterization; Isolates; Mycotoxins

INTRODUCTION

Zobo, a beverage made from hibiscus, is a drink that has been traditionally crafted through the processing of dried leaves from the roselle or sorrel plant (*Hibiscus sabdariffa*) (Aboagye, 2020). Numerous species of hibiscus can be found globally, particularly in regions such as the Caribbean and Africa. Fresh or dried calyces of *H. sabdariffa* are used in the preparation of herbal drinks, hot and cold beverages, fermented drinks, wine, jam, jellied confectionaries, ice

cream, chocolates, flavouring agents, puddings and cakes (Oboh and Elusiyan, 2004). The beverage is widely recognized for its numerous nutritional advantages. Studies have shown that extracts of *H. sabdariffa* have a lipid lowering activity which could prevent diseases like hyperlipidaemia and cardiovascular diseases (Ehsemokha, 2020). Analysis of quality attributes of Zobo drinks by Adesokan (2013) showed that incorporation of ginger and garlic extracts into the zobo drink could be an effective means of improving quality attributes of the drink. However, it is also believed to contribute to food poisoning in many individuals, primarily due to the methods employed in its production. It is likely that infections such as *Salmonellosis*, *Brucellosis*, *E. coli*, Tuberculosis, and *Staphylococcal* infections may be transmitted via Zobo, in addition to zoonotic and food-borne diseases (Ayandele, 2015; Healthlink, 2021) (Bristone *et al.*, 2018))

The increasing food demands of urban populations, coupled with economic challenges, have led to a significant rise in the number of food vendors in Nigeria. Despite the many benefits associated with street food, there are considerable health risks linked to this segment of the economy. Various studies indicate that food sold on the streets is susceptible to contamination from spoilage or harmful microorganisms (Adeleye *et al*., 2024, Nwaiwu *et al.*, 2020). Inadequate access to potable water, sanitation facilities, proper storage, and waste disposal systems at Zobo's preparation and sales locations has led to poor hygiene practices, potential exposure to contaminants, and an increased threat to public health (Omemu *et al.*, 2006). Many studies indicate that most outbreaks of foodborne acute gastroenteritis occur in places of mass production and distribution. Interestingly, the rising cases of food borne disease in Nigeria are not only linked to the misuse and abuse of agrichemicals on agricultural products, but are also related to people's poor food hygiene practices (Ezirigwe, 2018). In 2013, no fewer than thirty guests were hospitalized, and eight persons died after eating a suspected poisoned delicacy (Iyadi, 2015).

In developing nations such as Nigeria, effective regulation of the processing of street-vended foods remains a challenge. Furthermore, many vendors do not possess sufficient knowledge regarding proper food processing and handling techniques, which significantly heightens the risk of chemical and microbial contamination. Currently, the production of Zobo drink lacks both

mechanization and standardization, resulting in packaging or dispensing in nylon or plastic containers that are susceptible to contamination and pose an increased public health risk. Consequently, this study was conducted to identify and characterize the fungal species present in Zobo drinks that are prepared and sold locally.

Materials and Methods

Sample Collection

Branded and unbranded hawked Zobo drinks packaged in plastic bottles were purchased from ten different hawkers, two each from five locations in Eket metropolis, Akwa Ibom State. The samples were transported to the laboratory in the identical plastic containers utilized for their collection. In the laboratory, they were aseptically transferred into sterile specimen bottles and a portion subsequently stored in the refrigerator.

Media preparation

The media used for isolation and enumeration of fungi included Sabouraud Dextroxe Agar (SDA) and Malt Extract Agar. The composition and preparation of these media were according to the manufacturer's instructions.

Determination of the pH

The pH of the various samples of Zobo drink was determined using a pH meter (3505 pH meter, Jenway) by first preparing a standard buffer solution in a clean conical flask. One gram of standard buffer was dissolved in 100ml of distilled water. The pH probe was inserted into the standard buffer solution and calibrated to pH 4. The pH probe was then cleaned with distilled water and wiped dry before inserting into each Zobo drink sample measured into sterile beakers. The reading was taken when the pH meter read to the highest point and remained constant.

Determination of Total Fungal Count

This procedure was performed on a plate of Sabouraud Dextrose Agar (SDA) utilizing the pour plate technique. A ten-fold serial dilution of the sample was carried out by transferring 1 ml of the Zobo drink sample with a sterile pipette into 9 ml of distilled water contained in test tubes. After dilution, 1ml of the serially diluted sample at 10^{-3} dilutions was transferred into sterile petri dishes before pouring the media that was previously prepared into plates in duplicate. The

plates were swirled gently in different directions and allowed to solidify. The plates were then incubated at room temperature for 96 hours.

Characterization and Identification of Fungal Isolates

In order to obtain pure isolates, the fungal isolates were sub cultured into Malt Extract Agar, and isolated at room temperature for five days. The characterization and identification of fungi isolates were based on cultural and microscopic characteristics. The microscopic identification was done by preparing a wet mount of isolated colonies. 1 drop of Lactophenol was placed on a clean slide. A sterile inoculating needle was then used to pick a colony of the fungi from the subculture plate and smeared on the slide. The slides were covered with cover slips and observed under a light microscope using the X40 objective lens. The Burnette and Pankhaurst yeast identification scheme was used to identify the molds.

A full and accurate description of the fungal growth on the culture media was observed and recorded. Parameters like rate of growth, colony colour, and colour changes, colour of reverse colonies, odour and texture of surface were considered and compared with previous work done on fungi.

RESULTS

pH of Zobo Drink Samples

Results of the pH of Zobo drink samples purchased from various parts of Eket metropolis as indicated in Figure 1 below showed that the acidity of the samples increased after storage.

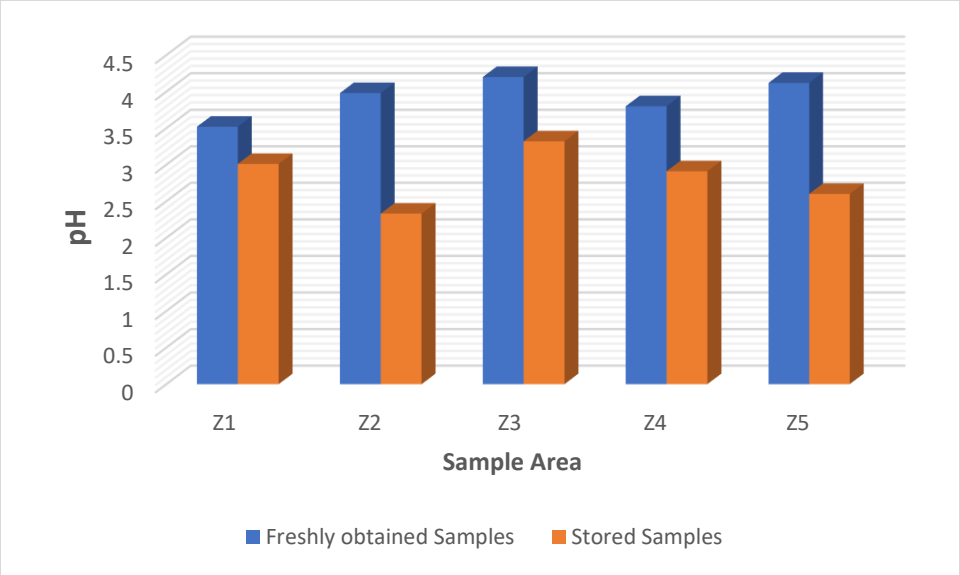

Figure 1: Mean Values of pH (Values represented as mean values of duplicate samples).

Total Fungal Counts

On Sabouraud Dextrose Agar, the average number of colonies for samples stored in the refrigerator ranged from 0.54 x 10^5 to 0.91 x 10^5 cfu/ml at 10^{-3} dilution while that of the freshly obtained samples ranged from 1.26 x 10^5 to 1.64x10^5 cfu/ml at 10^{-3} as show in figure 2.

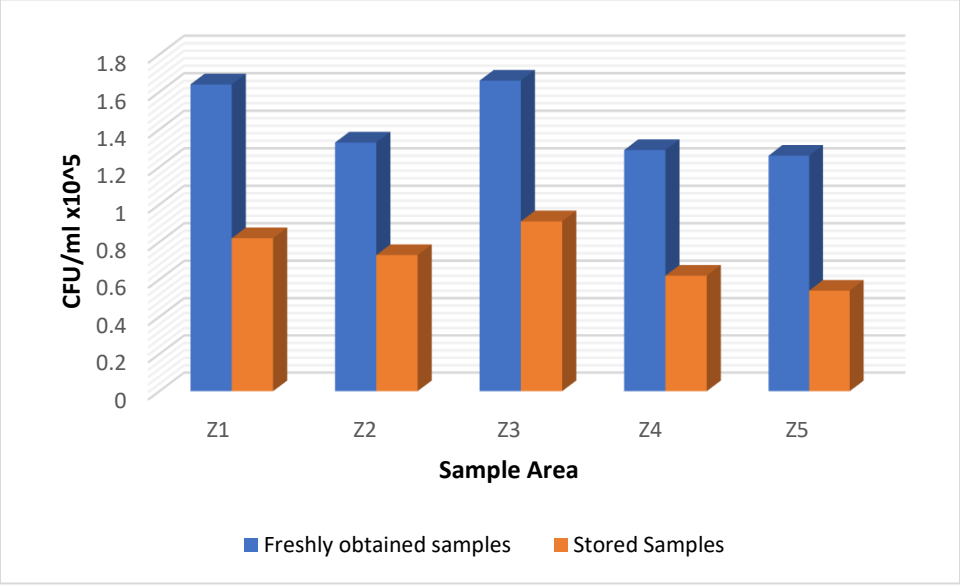

Figure 2: Mean Value of Total Fungal Counts

Characteristics of Fungi Isolated from Zobo Drink Samples

Macroscopic and microscopic analysis showed four fungal species which are *Penicillium spp., Aspergillus spp., Rhizopus spp. and Candida spp.* in the samples of Zobo drink examined. These were identified by their cultural characteristic and microscopic morphology.

Table 1: Cultural and Microscopic Characteristics of Fungal Isolates in Zobo Drink Samples

S/N	Cultural Characteristics	Microscopic characteristics	Probable Fungi
1.	Young colonies appearing blue-green to grey-green, becoming grey on maturation, with reverse white colour	Unbranched conidiophores arising from aerial hyphae; globose conidia	*Penicillium spp.*
2.	Greenish-yellow colour, fluffy and velvety texture, with cream reverse colour	Coarsely rough conidiophores, globose conidia and a septate hyphae	*Aspergillus spp*.
3.	Rapidly growing colonies, cotton-like texture, white initially and turns grey to yellowish brown on maturity, the reverse white to pale	Non-septate broad hyphae, brown and unbranched sporangiophores with may oval spores	*Rhizopus spp.*
4.	Macroscopically creamy, opaque, smooth and white colonies of	Globose to elongate yeast-like cells	*Candida* spp.

	different sizes		

Distribution and Frequency of Occurrence of the various Fungi Species

Figure 3 and 4 show the distribution of the identified fungi species from freshly obtained and stored Zobo drink samples respectively. A total of seventy-eight (78) characterized species were observed in the freshly obtained and fifty-one (51) in the stored Zobo drink samples.

The percentage frequency showed that *Aspergillus spp*. occurred most, with a frequency of 43.6% and 41.2% while *Rhizopus spp*. was the least in number (11.5%; 15.7%) (Figure 5).

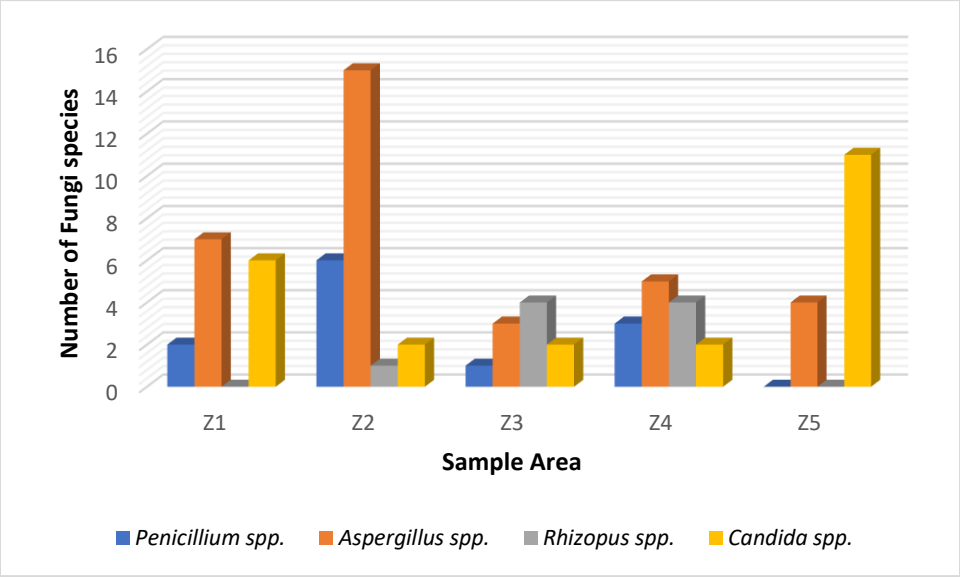

Figure 3: Distribution of Fungi Species in Freshly obtained Zobo drink samples

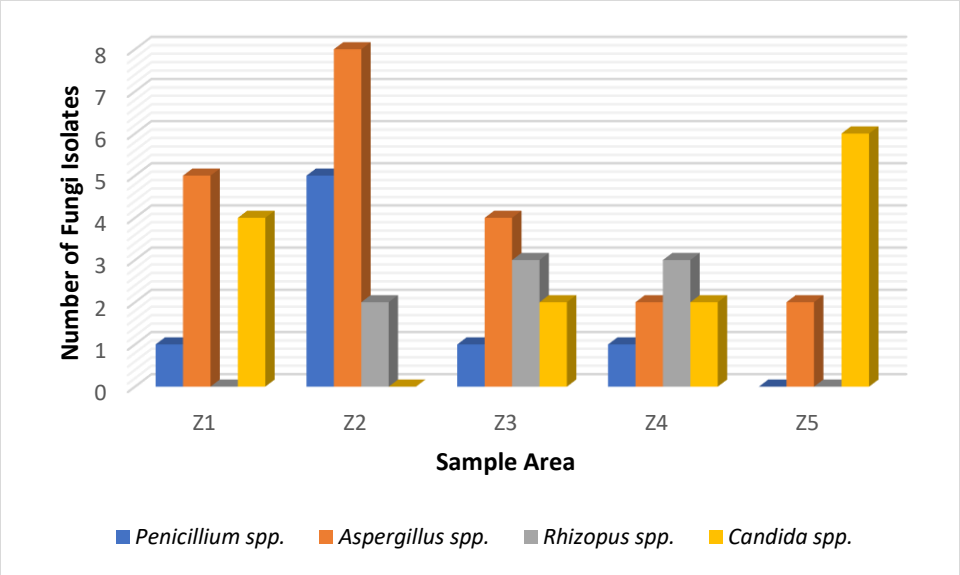

Figure 4: Distribution of Fungi Species in Stored Zobo drink samples

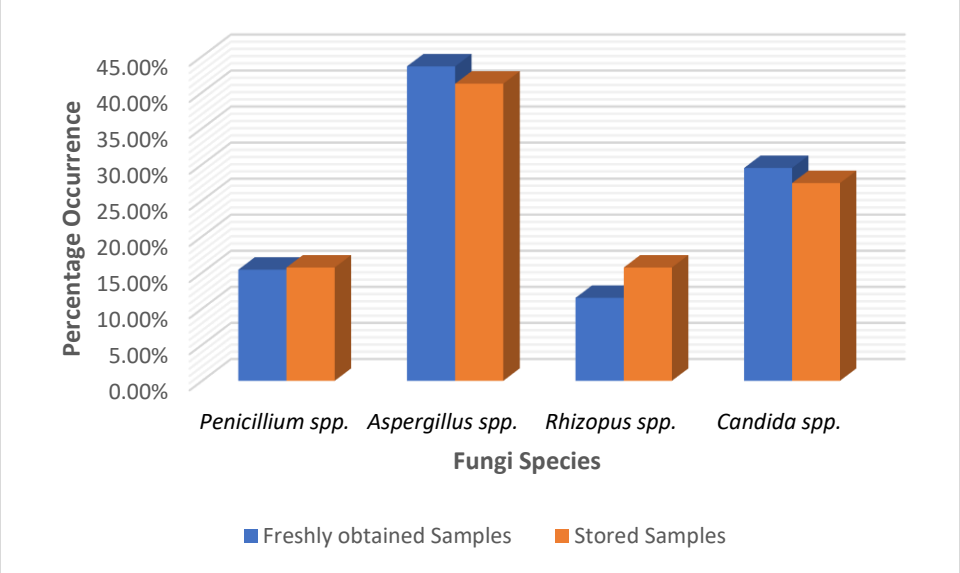

Figure 5: Comparison of Percentage Occurrence of Fungi Species in Freshly obtained and Stored Zobo drink samples

Discussion

The pH levels of the samples were found to be low (ranging from 2.33 to 4.12). The samples stored overnight had lower pH values. This aligns with the findings of Olayemi *et al.* (2011), who noted that the pH was on the lower end, thereby indicating and confirming the elevated acidity typically observed in Zobo drinks. This beverage is derived from a naturally acidic plant that

contains a variety of organic acids, including ascorbic, oxalic, tartaric, malic, and succinic acids (Ehsemokha, 2020). The elevated acidity may also serve to inhibit the proliferation of certain microorganisms that lack tolerance to such conditions (Jay, 1996).

The total fungi count for the freshly collected samples and those that were stored ranged from approximately 1.26 x 10^5 CFU/m^3 to 1.64 x 10^5 CFU/$m^3$and from 0.54 x 10^5 CFU/m^3 to 0.82 x 10^5 CFU/m^3, respectively. These values are considerably elevated when compared to the guidelines set by the American Conference of Governmental Industrial Hygienists (ACGIH), which recommends a total plate count of 100 to 1000 CFU/m^3 for food samples. Research conducted by Udensi *et al.* (2020) indicated a total fungal count ranging from approximately 1.2 x 10^4 to 3.1 x 10^4. Similarly, Braide *et al.* (2012) reported a total fungal count that varied from 1.0 x 10^7 to levels classified as Too Numerous To Count (TNTC).

The fungi characterization and identification revealed the presence of various yeast and molds. *Aspergillus spp., Candida spp. Penicillium spp.* and *Rhizopus spp.* were predominant. Nwaiwu *et al.,* (2020) reported *Aspergillus*, *Candida*, and *Penicillium* as commonly found fungi species in traditional beverages (soymilk, nono (fermented cow milk), tiger nut milk, yoghurt, kunu, zobo, palm wine, pito and brukutu) produced and sold across Nigeria. Other research results have validated the existence of various other microscopic organisms in Zobo including fungi (*Aspergillus flavus*, *F. oxysporum*, and *P. citrinum*); yeasts (*Saccharomyces cerevisiae*) and lactic acid bacteria, namely, *Lactobacillus planetarium* and *Streptococcus lactis* (Nwachukwu *et al.*, 2007; Braide *et al.*, 2009; Nwafor and Ikenebomeh, 2009; Ruiz-Ramírez *et al.*, 2015; Adeoye *et al.*, 2018). The presence of fungi may be attributed to the acidic nature of the samples since it has been observed that yeast and molds are capable of utilizing organic acids.

From this study, a total of seventy-eight (78) fungi strains were identified from the freshly obtained samples (43.6% *Aspergillus spp.,* 29.5% *Candida spp.* 15.4% *Penicillium spp.* and 11.5% *Rhizopus spp*). With increased acidity of the stored samples, the number reduced to fifty-one (51) (41.2% *Aspergillus spp.,* 27.4% *Candida spp.* 15.7% *Penicillium spp.* and 15.7% *Rhizopus spp*). The total counts varied from one sample to another. This agrees with the study of Onuorah and Odibo, 2018 who reported the presence of *Aspergillus niger* (23.77%), *Rhizopus stolonifer*

(19.48%), *Fusarium oxysporum* (8.57%), *Penicillium expansum* (17.14%), and *Saccharomyces cerevisiae* (31.04%) in Zobo samples purchased from hawkers in Ifite Awka, Nigeria. According to Udensi *et al.*, (2020) on microbiological quality of Zobo drink preserved with scent leaves, there were wide variations in the fungi population, with *Penicillium spp.* 6(16.2%) being the most predominant and occurring isolates, followed by *Aspergillus niger* and *Rhizopus spp.* at 4(10.8%) each.

The fungi species isolated in this study are capable of producing mycotoxins (Umaru *et al.*, 2014; Ayandele, 2015; Ismaiel, 2015). Mycotoxins of great concern for public health include aflatoxins (AF), ochratoxins (OT), trichothecenes, zearalenone (ZEN), fumonisins (F), tremorgenic toxins, and ergot alkaloids (Zain 2011). *Aspergillus spp.,* are known to produce aflatoxins which possess hepatotoxic and immune-suppressive effects and are also potential carcinogens. The types of infections caused by mycotoxigenic fungi depend on the type of mycotoxin, the concentration and length of exposure; as well as age, health, and sex of the exposed individual (Bennett and Klich 2003).

Penicillium species are diverse and widely distributed in the environment. The pathogenic species viz, *P. citrinum*, *P. chrysogenum*, *P. digitatum*, *P. expansum* and *P. marneffei* are commonly associated with humans/animals and the mode of infection being mostly through inhalation and sometimes ingestion (Wash *et al.,* 2004). *Penicillium* species also produce mycotoxins ochratoxin A, citrinin, patulin and penicillinic acid, which have cytotoxic effects on mammalian cell lines resulting in reduced cell viability depending on concentration and duration of exposure (Mwanza *et al.,* 2009; Oh *et al.*, 2012). Certain clinical strains of *Candida* have been associated with various infections, despite the presence of numerous nonpathogenic strains within the environmental flora. As a result of the different health effects of these filamentous fungi species and the metabolites they produce, this group of micro-organisms contribute to reduce the efficacy of the immune system of mammals upon continuous exposure to them (Joseph *et al.*, 2022).

As corroborated by other researchers, contamination of the Zobo drinks by fungi species noticed in this study may have occurred during cooling of the hot extract, addition of flavours

and sweeteners, dispensing of extract into bottles, utensils and water used during the post heating stages. Water used in processing has been identified as the major source of contamination of locally made drinks (Okeke *et al*., 2000; Joseph and Adogbo, 2015). The presence of fungi genera that produce most of the mycotoxins harmful to humans, animals, and plants may necessitate post-production testing of the drink to ensure food safety. Despite recent reports highlighting progress in antifungal therapy, it is important to acknowledge that the incidence of infections and the prevalence of antifungal resistance remain alarmingly high. Furthermore, the management of antifungal diseases does not suggest that effective control will be attainable in the near future. (Araj *et al.* 2015; Pellon *et al.* 2018).

In assessing the effect of some commonly used chemical preservatives (acetic acid and sodium benzoate), natural plant extracts (clove, garlic, ginger and lime) and pasteurization, on the elongation of shelf-life of Zobo beverage, Braide *et al.*, (2012) showed that there was a drastic reduction in microbial load as the effects of the preservative became evident. This suggests that adding preservatives to the locally prepared drink could prevent pathogenic microorganisms including fungi.

CONCLUSION

In Nigeria, Zobo is enjoyed by a diverse range of individuals. Its popularity has increased significantly, and it is available for purchase in various public locations, including schools, hospitals, workplaces, markets, and on the streets. The present study revealed the fungi status of Zobo drink produced and sold by vendors in Eket metropolis, Akwa Ibom State. The microbiological quality of fresh street-vended Zobo drink is concerning, as the majority of samples exceeded acceptable microbial limits, suggesting inadequate hygiene practices during handling, which poses a risk to consumers. Most of these organisms isolated from the Zobo drink are known to be the causative agents of food-borne gastroenteritis, immunotoxicity and cytotoxicity, therefore adequate hygienic practices must be observed during the preparation and handling of the Zobo drinks. Public awareness should also be created to emphasize the consequences of unhygienic conditions on human health.

Disclaimer (Artificial intelligence)

Option 1:

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

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