Mycotoxins in *khadi*, A Traditional Non-Cereal Based Alcoholic Beverage of Botswana

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**ABSTRACT:** Mycotoxin contamination is a major food safety drawback towards the commercialization of food products. The commercialization of *khadi*, a popular fermented alcoholic beverage of Botswana necessitates the investigation of the presence of mycotoxins. *Khadi* brewing involves the uncontrolled and unstandardized spontaneous fermentation of sun-dried *Grewia flava* fruits, which could be a source of mycotoxin-producing filamentous fungi (molds). This study sought to investigate the presence of mycotoxins producing fungi and mycotoxins in 18 samples of *khadi* collected in Central and Northern Botswana. *Periconia thailandica*, *Cladosporium cladosporioides*, *Aspergillus ochraceus*, *Phoma eupyrena*, *Selosphaeria turcica*, *Cladosporium sphaerospermum*, *Chaetomium longiciliata*, and *Flavodon ambrosius* were identified in 10 out of 18 *khadi* samples. Mycotoxins were detected using the Myco-10 Randox Evidence Investigator biochip kit and confirmed using a UPLC-ESI-MS/MS. Mycotoxins such as paxilline, ochratoxin A, ergot alkaloids, aflatoxin G1/G2, and zearalenone were detected using the Myco-10 Randox Evidence Investigator biochip kit. The Myco-10 results revealed that the mycotoxins in the *khadi* samples were lower than the regulatory limits set by FDA or European Commission. Confirmation of results using an UPLC-ESI-MS/MS system involved confirming selected mycotoxins (AFB1, DON, ZEA, FB1, FB2, FB3, NIV, and OTA) from selected *khadi* samples (Palapye 1, Palapye 2, Lethakane 2, Maun 3, Mmashoro 3, and Tonota 3). The UPLC results demonstrated that the aforementioned mycotoxins in the selected *khadi* samples were below the detection thresholds. The study shows that while fungal isolates were present, there is no to minimal danger/risk of exposure to toxic mycotoxins after consumption of *khadi*. Towards commercialization endeavors, the production process would necessitate minimal mycotoxin monitoring and product preservation but no detoxifying steps.

**KEYWORDS:** *Khadi*, fungal contamination, mycotoxins, spontaneous fermentation

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**Introduction**

*Khadi* is a popular traditional and non-cereal-based alcoholic beverage from Botswana, and it is usually prepared under non-standardized conditions. The brewing process involves uncontrolled spontaneous fermentation of sun-dried *Grewia flava* fruits and table sugar. Such uncontrolled spontaneous fermentation conditions expose the brew to mycotoxin-producing fungi contamination. Mycotoxins are a structurally diverse category of largely tiny molecular weight substances produced primarily by the secondary metabolism of various filamentous fungus, and in significant levels, they pose serious dangers to human health.¹² These human health implications include but are not limited to esophageal malignancies,² kidney mycotoxicosis,³ acute aflatoxicosis,¹ carcinogenic,² genotoxic,⁴ teratogenic,⁵,⁶ nephrotoxic,² and hepatotoxic⁴ and others.

Contamination by filamentous fungi is a problem at every stage of the fruit-harvesting process, from pre-harvest to post-harvest storage and processing. Some of the most common fungal genera known to produce mycotoxins are *Aspergillus*, *Alternaria*, *Fusarium*, and *Penicillium*.⁸,⁹ These mycotoxins often accumulate in the final alcoholic beverage. Concerning mycotoxins include aflatoxins, deoxynivalenol (DON), fumonisins, ochratoxins, citrinin, ergot alkaloids, patulin, and zearalenone (ZEN).¹⁰-¹³ Mycotoxicity of cereal based traditional alcoholic beverages made in beverages produced from substrates is well documented in the South African market.¹⁰,¹¹,¹⁴-¹⁶ Despite the fact that mycotoxins have been documented in African traditional alcoholic beverages, the mycotoxin data is lacking. Fatalities after drinking *khadi* have been reported¹⁷ and evidence shows death of 3 after consuming this alcoholic beverage.¹⁸ Unfortunately, the total of deaths per year for the past decade linked to *khadi* consumption are not available on formal record. Information on mycotoxins in *khadi* is crucial for commercialization as it is the first step to allow development of methods to prevent contamination. Despite the importance of keeping records on mycotoxins in foods processed at the household level due to the link between food safety and health, there is currently no law in Botswana governing mycotoxin regulation in traditional beverages. However, some products, including ready-to-eat food (10 ppb), peanuts (15 ppb), and milk (0.05 ppb), are regulated for their levels of aflatoxins.¹⁹ As such, this study
set out to conduct mycotoxin analyses on a popular beverage, *khadi*, as a critical step toward commercialization.

**Materials and Methods**

**Sample collection**

*Khadi* samples were randomly collected from local brewers in Botswana’s towns and villages of Tonota, Palapye, Serowe, Letlhakane, Mmashoro and Maun. Brewers in the southern part of Botswana were excluded because they brew *khadi* using tubers instead of *G. flava* fruits. During the sampling process, all the *khadi* samples were collected at a stage when the *khadi* was ready for consumption that is after completion of fermentation. Each sample was then transported to the laboratory at temperatures of 0°C to 4°C in a cooler box with ice packs and proceeded to −20°C until analyses.

**Isolation of fungi from *khadi* samples**

Hundred microliters of each aliquot of *khadi* were 10-fold serially diluted using 1 mL phosphate buffered saline (PBS, g/L: NaCl 8.0, KCl 0.2, disodium phosphate 1.44, and potassium phosphate 0.24), pH 7 and kept on ice. The dilutions (up to 10^−6) were then spread plated on YPD agar (2% glucose, 1% yeast extract, 2% peptone, and 1.5% agar) supplemented with 100 µg/mL of streptomycin, penicillin, and ampicillin. The yeasts extract, 2% peptone, and 1.5% agar) supplemented with 100 µg/mL of streptomycin, penicillin, and ampicillin. The plates were incubated at 30°C for 5 days. The fungal colonies on the YPD plates were then cut and transferred to malt extract agar (MEA) (Oxoid, England) and potato dextrose agar plates (PDA) (Difco, Detroit, MI) to get pure cultures which were then incubated at 30°C for 5 days. For further study, the purified cultures were stored in 25% glycerol at −80°C.

**Identification of fungi from *khadi* samples**

For identification of fungal isolates, we first extracted gDNA using the microwave method. In brief, the fungal mat was rinsed with 1 mL TE buffer, centrifuged, and lysed using 100 µL TE buffer and 50 µL 10% SDS. The lysate was incubated for 30 minutes at 65°C and centrifuged to remove the supernatant. The cell pellet was placed in a microwave oven and heated twice for 1 minute at 900 W. The pellet was then re-suspended in 200 µL TE buffer with one volume of phenol:chloroform:isoamyl alcohol (25:24:1) for 15 minutes. The aqueous phase was recovered by centrifugation; DNA was precipitated with 95% ethanol and centrifuged at 12,000 rpm for 20 minutes. DNA was then rinsed with 1 mL 70% ethanol and re-suspended in 200 µL deionized water.

Two microliters of the suspension was then used as a template for PCR amplification of a 560 bp DNA fragment of the D1/D2 region of the 26S rDNA using universal PCR primers, NL1 (5′-GCATATCAATAAGCGGGAGGAAAAG-3′) and NL4 (5′-GGGCTCCGTGTGTAGGACCAGG-3′) as reported elsewhere. PCR amplification was performed using ProFlex PCR Systems (Applied Biosystems, USA) in a 20 µL reaction volume containing 2.5 µL 10× PCR buffer, 2.0 µM of each primer, 0.2 mM dNTPs, and 1.25 U *Taq* DNA polymerase (Takara Bio Inc., Japan) using the following cycling conditions: initial denaturation at 98°C for 3 minutes; 38 cycles of denaturation (98°C for 15 seconds), annealing (54°C for 1 minute), and extension (72°C for 1 minute); a final extension step at 72°C for 7 minutes and held at 4°C until required. Sterile water was used instead of template DNA for negative controls that were run alongside test samples. All amplicons were purified using a QiAquick PCR product purification kit (Qiagen, GmbBH, Germany) according to manufacturer’s instructions. The amplicons were sequenced by Inqaba Biotech (Pretoria, South Africa). SnapGene® Viewer software ver. 4.2.11 (GSL Biotech) sequence editing tool was used to generate contiguous sequences (http://www.snapgene.com). Species identification was done by using a BLAST nucleotide sequence analysis tool (https://blast.ncbi.nlm.nih.gov/BLAST.cgi; PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome) as well as using pairwise identification tool available on the Westerdijk Fungal Biodiversity Institute (CBS-KNAW) strain database (http://www.westerdijk institute.net/).

**Mycotoxins detection from *khadi* using the Randox Evidence Investigator**

Simultaneous semi-quantitative screening of mycotoxins in feed was carried out using the Myco 7 biochip array kit (EV4065; Randox Food Diagnostics, Crumlin, UK). The kit contains multianalyte biochips, assay diluent, conjugate diluent, multianalyte conjugate, a set of multianalyte calibrators (spanning the range of each assay), multi-analyte controls, signal reagent, washing buffer, calibration disk, and barcodes. The biochips were supplied in carriers (3 × 3 biochips per carrier), and a carrier handling tray was provided with the system that allows the simultaneous handling of 6 carriers (54 biochips). Data were generated and processed with the semi-automated benchtop biochip analyzer Evidence Investigator (EV3602; Randox Food Diagnostics, Crumlin, UK).

**Extraction of mycotoxins from *khadi* samples (Acetonitrile method)**

To extract mycotoxins from the samples, a total of 2.5 mL of each *khadi* sample was mixed with 20 mL pure acetonitrile (Sigma-Aldrich, USA) in a 50 mL centrifuge tube. Sodium chloride (2 g) and magnesium sulfate (0.25 g) were added to the mixture. The resultant mixture was vortexed for 3 minutes followed by centrifugation at 4000 rpm for 10 minutes at 4°C. The supernatant was collected into a new 2 mL centrifuge tube and the pellet was discarded.

**Randox Evidence Investigator Myco-10 calibration.** The Randox Evidence Investigator was calibrated based on the nine-point calibration curve using Randox Evidence Investigator Myco-10 calibrator (Randox Food Diagnostics) (https://www.randox.com/evidence-investigator-multiple-industries/)
which covered the calibration range of all assays. In brief, calibration data was used to calculate the linearity, limit of detection (LOD) and limit of quantification (LOQ) of the calibration process. The LOD is the detection limit of an analytical procedure and is the lowest concentration of analyte in a sample that can be detected while the LOQ is the smallest concentration of an analyte that can be reliably measured by an analytical procedure.

To cover worldwide guidance limits, the overall sample dilution factor could be selected at lower and higher levels (dilution factor 20 for sensitive detection and 250 for monitory level, respectively). The multi-analyte calibrators included all the analytes used for the standardization of each of 7 competitive immunoassays on the biochip at 9 different concentration levels, with the following typical calibration ranges for sensitive detection and monitory level, respectively: 0 to 3.7 and 0 to 46.25 ppb expressed as aflatoxin B1 equivalence for the aflatoxin B1 assay, 0 to 20 and 0 to 250 ppb expressed as aflatoxin G1 equivalence for the aflatoxin G1 assay, 0 to 2000 and 0 to 25 000 ppb expressed as DON equivalence for the DON assay, 0 to 80 and 0 to 1000 ppb expressed as fumonisin B1 equivalence for the fumonisins assay, 0 to 16 and 0 to 200 ppb expressed as ochratoxin A equivalence for the ochratoxin A assay, 0 to 80 and 0 to 1000 ppb expressed as T-2 toxin equivalence for T-2 toxin assay, and 0 to 40 and 0 to 500 ppb expressed as zearalenone equivalence for the zearalenone assay.

Mounting of samples onto Myco-10 biochip. To begin the sample mounting process, 150 µL of diluted wash buffer was pipetted into each of the 9 biochip wells (Myco-10 biochip, Randox Diagnostics, UK). Fifty microliters of each of the extracted samples was added to the appropriate biochip well. Mixing of the samples and the wash buffer was done by gently tapping the edges of the handling tray. The trays were placed on a thermal shaker for 30 minutes at 370 rpm and 25°C. After incubation, a working solution was prepared (provided in the Myco-10 kit) of 100 µL which was pipetted into each biochip well. The biochip was then incubated at 25°C for 60 minutes at a shaking speed of 370 rpm. The biochip was then washed 4 times by pouring 375 µL of wash buffer to each biochip well. Another wash cycle was carried out 4 times and the biochips were then vortexed for 60 seconds and hand rolled for 10 minutes. After washing, the mixture was centrifuged for 10 minutes at 3000 rpm to complete the extraction. The mycotoxin quantification (Sections 2.4.3 and 2.4.4) and spikings were done as stated above.

Myco-10 biochip imaging. Each biochip carrier was imaged individually and biochips awaiting imaging were protected from light by covering them with a lint free paper towel. Each biochip well was tapped onto a lint free paper towel to remove any residual wash buffer. After tapping, the biochip wells were filled with wash buffer to avoid drying out and left to soak until directly prior to imaging.

Myco-10 biochip imaging. Each biochip carrier was imaged individually and biochips awaiting imaging were protected from light by covering them with a lint free paper towel. Each biochip well was tapped onto a lint free paper towel to remove any residual wash buffer. A signal reagent (provided in the Myco-10 kit) volume of 250 µL was added to each biochip well and covered to protect from light for 2 minutes. The biochip carrier was placed into the Evidence Investigator (EV3602; Randox Food Diagnostics, Crumlin, UK), images from each well were captured and mycotoxins per well quantified. In the Evidence Investigator, the degree of binding is determined using a chemiluminescent light source and then quantified using a charge coupled device (CCD) camera and imaging system. The Randox Evidence Investigator detects and quantifies 10 mycotoxins per sample (paxilline, fumonisin, ochratoxin A, aflatoxin G1/G2, ergot alkaloids, diacetoxyisocupenol, deoxynivalenol, aflatoxin B1/B2, and zearalenone). The signal from sample chips was compared to the signal from the control (calibrators) to obtain a semi-quantitative result.

Efficiency and accuracy of the extraction methods. The extraction method was confirmed for accuracy and efficiency by spiking 3 khadi samples from 3 different sampling locations with known concentrations of AFB1 (0.1 ppb) and FB1 (1 ppb). The Myco-10 extraction method was also used as instructed by the manufacturer (Randox Diagnostics, UK) and compared with the above extraction method (Section 2.4.1). The Myco-10 extraction method involved taking 5 mL of the khadi sample and adding 25 mL of acetonitrile: methanol: water (50:40:10). The mixture was then vortexed for 60 seconds and hand rolled for 10 minutes. After rolling, the mixture was centrifuged for 10 minutes at 3000 rpm to complete the extraction. The mycotoxin quantification (Sections 2.4.3 and 2.4.4) and spikings were done as stated above.

Mycotoxins detection from khadi using the UPLC-ESI-MS/MS

Sample preparation. To prepare the samples for mycotoxins analysis using the UPLC, all the samples were diluted in duplicates with 75% water: 25% methanol solvent into respective 2 mL microcentrifuge tubes and centrifuged for 5 minutes at 13 000 rpm. The supernatant was transferred to a 2 mL microcentrifuge tube and the pellet discarded. One milliliter of diluted sample was then aliquoted into an analysis vial.

Standard solutions. The following mycotoxin standards and metabolites were purchased from Biopure (Tulln, Austria): fumonisin B1, fumonisin B2, fumonisin B3, ochratoxin A, aflatoxin B1, nivalenol, diacetoxyisocupenol, and zearalenone. Acetonitrile (ACN), water and methanol (MeOH) were purchased from Biosolve (Valkenswaard, Netherlands) and formic acid (FA) from Merck (Amsterdam, Netherlands). The working solutions were prepared as follows: fumonisins solution containing FB1, B2, and B3 (each at 5 µg/mL) was diluted in acetonitrile/water (50/50 v/v) and stored 4°C; aflatoxin B1 (1 µg/mL), ochratoxin A solution (1 µg/mL), and the other mycotoxins solution containing nivalenol, deoxynivalenol, zearalenone (each at 10 µg/mL) were diluted in acetonitrile and were stored –20°C.

Mycotoxin quantification. A Waters Acquity ultra performance liquid chromatography (UPLC) was coupled to a Xevo triple quadrupole tandem mass spectrometer (MS/MS) (Waters,
Table 1. Identity of filamentous fungi isolates from the khadi samples based on D1/D2 domains of 26S rDNA.

| SAMPLE COLLECTION LOCATION | ISOLATE NAME                        | ACCESSION NUMBER | IDENTITY (%) |
|----------------------------|-------------------------------------|------------------|--------------|
| Serowe 1                   | Cladosporium sphaerospermum         | KY873376.1       | 99           |
| Serowe 3                   | Periconia thailandica               | KY753888.1       | 99           |
| Palapye 2                  | Cladosporium cladosporioides        | KM246047.1       | 99           |
| Palapye 3                  | Aspergillus ochraceus               | KX958037.1       | 99           |
| Maun 1                     | Phoma eupyrena                      | JF766684.1       | 99           |
| Maun 1                     | Setosphaeria turcica                | LT715640.1       | 99           |
| Maun 2                     | Cladosporium sphaerospermum (2)     | KX973376.1       | 99           |
| Mmashoro 2                 | Chaetomium longiciliatia            | KP336823.1       | 99           |
| Tonota 3                   | Aspergillus ochraceus (2)           | KX958037.1       | 99           |

The number in the bracket shows strains with the same accession number from the same location.

Results and Discussion

Khadi contaminated with mycotoxin-producing fungi

To determine if there was filamentous fungal contamination, khadi samples were grown on filamentous fungal isolation media. The findings revealed that a majority of the samples were contaminated with a variety of filamentous fungal species of phylogenetically diverse background (Supplemental Figure S1). *Aspergillus ochraceus*, *Cladosporium sphaerospermum*, *Cladosporium cladosporioides*, *Phoma eupyrena*, and *Setosphaeria turcica*, to name a few, were among the 14 filamentous fungal isolates found (Table 1 and Supplemental Figure S1). The predominant isolates were *C. sphaerospermum* (Serowe and Maun), *A. ochraceus* (Palapye and Tonota), and *Flavodon ambrosius* (Mmashoro) (Table 1), accounting for 21.4% of the total isolates.

The introduction of filamentous fungi to khadi might be through the fruits or as contamination from the brewers or brewing equipment. *Khadi* that has visible filamentous fungal growth on it may have a shorter shelf life or become unfit for human consumption. However, the presence of filamentous fungi hardly never indicates the presence of mycotoxins. The presence of these filamentous fungal isolates on the khadi samples may indicate that the food item possesses the requisite ideal conditions, such as high water activity, the presence of sugars, and the presence of organic acids capable of decreasing pH 8. Notably, the presence of filamentous fungal isolates in beverages such as *Aspergillus ochraceus* can signal the potential existence of mycotoxins. The presence of *Aspergillus ochraceus* therefore, is of extremely concerning because it has been shown to produce aflatoxins and ochratoxins which are harmful to mammalian cells and can cause cancer, nephrotoxicity, hepatotoxicity, and teratogenic effects. Fusariotoxins have terpenoid biosynthesis route as one of...
their characteristics (eg, trichothecenes like toxin T2 and deoxynivalenol, the main precursors of which are mevalonic acid and farnesyl pyrophosphate). Other isolates of interest that were identified were \textit{C. sphaerospermum} and \textit{C. cladosporioides}, which are filamentous fungi previously isolated from flowers of edible fruit plants as well as being isolated from honey, dairy products and grapes prior to harvesting.\textsuperscript{8,25-27} The \textit{Cladosporium} species have been described in the past as mycotoxin-producing and possibly pathogenic fungi that are frequently found in outdoor settings.\textsuperscript{28} The subsequent mycotoxin studies were necessary because the filamentous fungi were deemed to be a potential signal of mycotoxin presence in the \textit{khadi} samples.

Mycotoxins detection from \textit{khadi} using the Randox Evidence Investigator

\textbf{Randox Evidence Investigator Calibration.} The presence of fungal isolates (Table 1 and Supplemental Figure S1) in the \textit{khadi} samples prompted the examination of the beverage for mycotoxins. Quantification of mycotoxin was done with the Randox Evidence Investigator. To optimize the sensitivity of the method and assess its accuracy, the Randox Evidence Investigator was calibrated using the supplied calibrators of a known concentration. The acquired data was then used to provide information on the limits of detection (LODs) and limits of quantification (LOQs). LOD and LOQ are terms used to describe the smallest concentration of an analyte that can be reliably measured by an analytical procedure with an acceptable degree of accuracy and precision. The LOD and LOQ were calculated based on 3- and 10-fold signal to noise (S/N) ratios, respectively (Table 2). The LOD ranged from 0.13 to 101.33 ppb while the LOQ ranged from 0.43 to 337.78 ppb with DON having the highest LOD and LOQ. The linearity was established by the calibration curve based on the expected values and actual values from calibrators. The coefficient correlations $R^2$ ranged from 0.97 to 0.99 thus showing good linearity (Table 2) hence validating results from the Evidence Investigator (Supplemental Figure S2).

Three samples from 3 different sampling areas were then spiked with a known concentration of AFB1, and FB1 to test the efficiency of the acetonitrile method (Section 2.4.1) and the Myco-10 method (Section 2.4.5) according to the manufacturer’s instruction. The recovery of the samples ranged from 45% to 107% using the acetonitrile method where the best recovery was observed on FB1 and AFB1. Table 2 shows that the recovery challenge is not with the biochip because the quantification of the control standards read values close to the true concentrations as stated in the manual provided by the manufacturer. This is further confirmed by the $R^2$ of the sample calibrators (Supplemental Figure S2). The results in Table 3 also show that the Myco-10 extraction method was not suitable for use in this experiment. The Myco-10 extraction method was based on the use of chemicals namely acetonitrile, methanol and water as the extraction solvents and no organic salts. The acetonitrile method was more effective as it counteracted the presence of water in \textit{khadi} by using organic salts to bind to the water molecules.\textsuperscript{29} This step was missing in the Myco-10 extraction method resulting in the negative values in Table 3. Due to the lack of meaningful

| COMPOUND | LINEAR EQUATION | $R^2$ | SD  | LOD  | LOQ  |
|----------|-----------------|------|-----|------|------|
| PAX      | $Y = 0.9634x + 0.0252$ | 0.99 | 6.48 | 20.18 | 69.39 |
| FUM      | $Y = 1.0061x - 0.1004$ | 0.97 | 0.68 | 2.03  | 6.77  |
| OTA      | $Y = 1.0088x - 0.0131$ | 0.98 | 0.27 | 0.81  | 2.70  |
| AFG1     | $Y = 0.9883x - 0.0103$ | 0.98 | 0.33 | 1.01  | 3.38  |
| EA       | $Y = 1.0056x - 0.1973$ | 0.97 | 4.49 | 13.41 | 44.70 |
| DAS      | $Y = 1.0816x - 0.4864$ | 0.97 | 7.33 | 20.33 | 67.76 |
| DON      | $Y = 1.0907x - 2.4123$ | 0.98 | 36.84| 101.34| 337.78|
| T2       | $Y = 1.0935x - 0.0721$ | 0.98 | 1.47 | 4.04  | 13.47 |
| AFB1     | $Y = 1.0769x - 0.0023$ | 0.98 | 0.05 | 0.13  | 0.43  |
| ZEA      | $Y = 1.0766x - 0.0459$ | 0.99 | 0.72 | 2.02  | 6.73  |

Abbreviations: AFB1/B2, aflatoxin B1/B2; AFG1/G2, aflatoxin G1/G2; DAS, diacetoxyscirpenol; DON, deoxynivalenol; ERG, ergot alkaloids; FUM, fumonisins; OTA, ochratoxin A; PAX, paxilline; ZEA = zearalenone.

| SAMPLE                  | FB1 (1 PPM) (%) | AFB1 (0.1 PPM) (%) |
|-------------------------|-----------------|-------------------|
| Maun 1 (ACN method)     | 91.40           | 53.10             |
| Mmashoro 2 (ACN method) | 103.70          | 45.80             |
| Serowe 1 (ACN method)   | 107.30          | 61.90             |
| Serowe 1 (Myco-10 method)| −98.2          | −75               |

Table 2. The calibration of the Randox Evidence Investigator using the control toxins provided with the kit to determine the LOD and LOQ of each toxin.

Table 3. The product recovery of the spiked samples using the above extraction method.
results from the Myco-10 method, it was deemed unsuitable for use in this study.

**Mycotoxins from khadi.** After the optimization of the acetoni-trile method, the *khadi* samples were analyzed for presence of mycotoxins. All the samples contained fumonisin, ochratoxin A, diacetoxyscirpenol, and zearalenone (Table 4). The least detected toxins from the brews were paxilline (detected from Mmashoro 1, Mmashoro 2, Palapye 1, Palapye 3, Serowe 1, and Serowe 3), deoxynivalenol (detected from Maun 1, Maun 2, Serowe 1, Tonota 1, Tonota 2, and Tonota 3) and aflatoxin B1/B2 (detected from Mmashoro 3, Serowe 1, and Tonota 2). The Serowe 1 sample contained all 10 mycotoxins. The quantification of mycotoxin shows that DAS was the most abundant (ranged from 3.47 to 33.79 ppb) and aflatoxin B1/B2 was the least abundant (ranged from 0 to 0.049 ppb). Other toxins included PAX, which ranged from 0 to 0.16 ppb, FUM which ranged from 0.14 to 2.27 ppb, OTA which ranged from 0.33 to 3.84 ppb, AFG1/G2 which ranged from 0.12 to 1.02 ppb, ERG which ranged from 0 to 4.93 ppb, DON which ranged from 0 to 21.86 ppb, and ZEA which ranged from 0.004 to 0.49 ppb.

Mycotoxins such as zearalenone have been detected from traditional opaque beers of Botswana 13 while others such as ochratoxin A, deoxynivalenol, fumonisin B1, and fumonisin B2 have been detected in various European beers.30 In another investigation on the South African maize-based alcoholic beverage *umqombothi*, the authors detected mycotoxins such as T2,
DON, fumonisin, zearalenone, and aflatoxins with evidence pointing at the raw material or substrate as the source of the mycotoxins.\textsuperscript{15,16} Both dried and fresh fruits tend to have mycotoxins.\textsuperscript{31,32} Mycotoxins accumulate during the harvesting, drying and storage due to conditions that favor mycotoxin accumulation.\textsuperscript{33,34} The \textit{A. ochraceus} produces ochratoxin A, which was detected in all the \textit{khadi} samples in this work. The detection of other mycotoxins such as aflatoxins is characteristic of other fungi such as \textit{A. flavus}, \textit{A. parasiticus}, \textit{A. bombycis}, \textit{A. ochraceus}, \textit{A. nomius}, and \textit{A. pseudotamari}.\textsuperscript{1,9,24,35-37} In this work, only \textit{A. ochraceus} was isolated from \textit{khadi} and thus \textit{A. ochraceus} could be the only possible source of aflatoxins detected in \textit{khadi}.

Different countries have different mycotoxins limitations.\textsuperscript{38} The Food and Drug Administration (FDA) allows aflatoxins to be at a maximum level of 4 to 20 ppb while European Commission (EC) has a rigorous restriction of 0.5 to 10 ppb per food item\textsuperscript{5} yet all the \textit{khadi} samples were below the permissible range. The FDA also has limits on fumonisins (0.2-4 ppb), aflatoxins (20 ppb), ZEA (20-200 ppb), and T2 (80-100 ppb).\textsuperscript{5} The \textit{khadi} samples were all below these limits, implying that they may not cause any major health risks when consumed (Table 4). These findings imply that there is no risk of mycotoxin poisoning after consumption of \textit{khadi}.

### Table 6. Mycotoxin detection through UPLC-ESI-MS/MS from the \textit{khadi} samples with the FDA and EC limits.

| MYCOTOXIN | SAMPLE | TOXIN QUANTIFICATION | FDA LIMIT (PPB) | EC LIMIT (PPB) |
|-----------|--------|-----------------------|----------------|----------------|
| AFB1      | Palapye 1, Palapye 2, Letlhakane 2, Mmashoro 3, Maun 3, and Tonota 3 | ND            | <20            | 2              |
| NIV       | Palapye 1, Palapye 2, Letlhakane 2, Mmashoro 3, Maun 3, and Tonota 3 | ND            |                |                |
| DON       | Palapye 1, Palapye 2, Letlhakane 2, Mmashoro 3, Maun 3, and Tonota 3 | ND            | 1000           | 1 ppb BW/day   |
| ZEA       | Palapye 1, Palapye 2, Letlhakane 2, Mmashoro 3, Maun 3, and Tonota 3 | ND            | 0.25 ppb BW/day| 20-200         |
| OTA       | Palapye 1, Palapye 2, Letlhakane 2, Mmashoro 3, Maun 3, and Tonota 3 | ND            | Not set        | 2000           |
| FB1       | Palapye 1, Palapye 2, Letlhakane 2, Mmashoro 3, Maun 3, and Tonota 3 | ND            | 2000-4000      | 200-4000       |
| FB2       | Palapye 1, Palapye 2, Letlhakane 2, Mmashoro 3, Maun 3, and Tonota 3 | ND            | 2000-4000      | 200-4000       |
| FB3       | Palapye 1, Palapye 2, Letlhakane 2, Mmashoro 3, Maun 3, and Tonota 3 | ND            | 2000-4000      | 200-4000       |

AFB1, aflatoxin B1; BW/day, body weight per day; DON, deoxynivalenol; FB1, fumonisin B1; FB2, fumonisin B2; FB3, fumonisin B3; NIV, nivalenol; OTA = ochratoxin A; ZEA = zearalenone.

The mycotoxins levels in the \textit{khadi} samples are below the FDA and EC regulation standards. The results from the Myco-10 biochip detected mycotoxins from all the \textit{khadi} samples (Table 6) but the UPLC-ESI-MS/MS showed that the mycotoxins in the samples were below limit of detection (Table 6). These findings indicate that \textit{khadi} can be consumed with little danger of mycotoxin poisoning. If mycotoxins are present at quantities exceeding set regulatory limits, they can have major health consequences.\textsuperscript{1,2} Although the \textit{khadi} samples were contaminated with filamentous fungi (Table 1), the absence of mycotoxins is not surprising given that literature implies that conditions conducive to fungal growth and development are not conducive to mycotoxin production.\textsuperscript{8} Similarly, the absence of filamentous fungi does not always imply the absence of mycotoxins, as mycotoxins might persist after the disappearance of fungi.\textsuperscript{39}

### Conclusion
Mycotoxin contamination research is crucial step toward commercialization of innovative food products. \textit{Khadi}, like most spontaneously fermented beverages, was discovered to harbor fungal isolates. Although some of isolates are known to

\begin{table}[h]
\centering
\caption{Confirmation of presence of mycotoxins in \textit{khadi} using the UPLC-ESI-MS/MS}
\begin{tabular}{|l|l|l|l|l|}
\hline
MYCOTOXIN & SAMPLE & TOXIN QUANTIFICATION & FDA LIMIT (PPB) & EC LIMIT (PPB) \\
\hline
AFB1 & Palapye 1, Palapye 2, Letlhakane 2, Mmashoro 3, Maun 3, and Tonota 3 & ND & <20 & 2 \\
NIV & Palapye 1, Palapye 2, Letlhakane 2, Mmashoro 3, Maun 3, and Tonota 3 & ND & 1000 & 1 ppb BW/day \\
DON & Palapye 1, Palapye 2, Letlhakane 2, Mmashoro 3, Maun 3, and Tonota 3 & ND & 0.25 ppb BW/day & 20-200 \\
ZEA & Palapye 1, Palapye 2, Letlhakane 2, Mmashoro 3, Maun 3, and Tonota 3 & ND & Not set & 2000 \\
OTA & Palapye 1, Palapye 2, Letlhakane 2, Mmashoro 3, Maun 3, and Tonota 3 & ND & 2000-4000 & 200-4000 \\
FB1 & Palapye 1, Palapye 2, Letlhakane 2, Mmashoro 3, Maun 3, and Tonota 3 & ND & 2000-4000 & 200-4000 \\
FB2 & Palapye 1, Palapye 2, Letlhakane 2, Mmashoro 3, Maun 3, and Tonota 3 & ND & 2000-4000 & 200-4000 \\
FB3 & Palapye 1, Palapye 2, Letlhakane 2, Mmashoro 3, Maun 3, and Tonota 3 & ND & 2000-4000 & 200-4000 \\
\hline
\end{tabular}
\end{table}
produce mycotoxin, our findings suggest that there are minimal risks of mycotoxins toxicity associated with khadi. Using 2 distinct procedures, all of the mycotoxins were either below regulatory levels or undetectable. This results from this study thus show that khadi mycotoxin levels are within safe limits for human consumption.

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Supplemental Material
Supplemental material for this article is available online.

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