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Reduction in growth and aflatoxin production in orange juice inoculated with *Aspergillus flavus* using *aframomum danielli*

Adegoke G. O.[1], Adekoyeni O. O.[2]*, Akinoso R.[1] and Olapade A. A.[1]

[1]Department of Food Technology, University of Ibadan, Nigeria.
[2]Federal University Dutsin-Ma, Katsina, Nigeria

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The survival and production of aflatoxin by *Aspergillus flavus* introduced into pasteurized (85°C;15 min) and unpasteurized orange juice treated with *Aframomum danielli* (local preservative) extracts at 0, 1, 2, 3, 4, 5 and 6% concentrations were investigated at 3 and 7 days of storage on shelf (28±2°C). There was slight increase in pH on day 3 which was more prevalent on day 7(4.77-6.85 and 6.07-7.37) for the pasteurized and unpasteurized orange juice respectively. There was no significant difference (p≥0.05) in growth and aflatoxin levels on day 3, however, luxuriant growth of *A. flavus* (0.63-1.42 g and 0.52-0.68 g) and aflatoxin production (29-156 ng/ml and 5.40-63.00 ng/ml) were recorded on day 7 respectively. The pasteurized sample (0%; control) of *A. danielli* had the highest growth and aflatoxin level followed by the sample with 6% extract with the same trend in unpasteurized juice. *A. danielli* at very low concentration showed effective inhibition of *A. flavus* growth and produced low levels of aflatoxin. 1% showed effective inhibition of *A. flavus* growth in both while 2 and 3% produced low levels of aflatoxin for pasteurized and unpasteurized products respectively.

Key words: *Aframomum danielli, Aspergillus flavus,* aflatoxin, orange juice, pasteurised.

INTRODUCTION

Sweet orange (*Citrus sinensis*) is a common fruit in warm climates and found to be rich in carbohydrates, pectin, ascorbic acid, flavonoids and essential oils. The healing properties of orange have been associated with a wide phytonutrient compounds such as flavonones, anthocyanins, hydrocinamic acids and a variety of polyphenols (Shahnawaz et al., 2013). The fruit is commonly peeled and eaten fresh or squeezed for its juice (Crowel, 1999). The peel of citrus fruit serves as natural protectant that prevents microbiological contamination of the interior flesh. Therefore, removing the peel eliminates this protective layer and subjects the edible portion to potential microbial invasion and spoilage.

Fruit juice is usually pasteurised to inactivate microbes and retard biochemical reactions which results to food spoilage. Pasteurization is mild heat treatments usually below 100°C, used to extend the shelf life of foods. The process destroys heat sensitive microorganisms such as non spore forming bacteria, yeasts, and moulds but there

*Corresponding author. E-mail: oludareadek@yahoo.com, Fax: +234 805 197 5288.

is some concern that some strains of pathogenic organisms may survive pasteurization (Aworh, 2003).

Aflatoxin is the most common mycotoxin found in foods with *Aspergillus flavus* as the major producer (Bradburn et al., 1993). Exposure to aflatoxin is wide spread in West Africa and has been implicated in carcinogenicity, mutagenicity, teratogenicity, hepatoxicity and aflatoxicosis (Bankole and Adabanjo, 2003). Bankole (2005) isolated 12 species of fungi from rotting sweet orange, 8 isolates were known to be producers of toxin while 7 of *A. flavus* obtained were aflatoxigenic producing primarily aflatoxin B1. The use of chemicals to enhance the safety of fruit juice is of great interest to the fruit industry. However, reports on the harmful effects of these synthetic preservative additives and interest of consumers in fresh foods, with reduced or no content of chemical preservations call for the research into the use of naturally derived compounds as alternative to synthetic additives.

The use of natural preservatives is a promising alternative to chemical methods in extending shelf life of products (Fasoyiro and Adegoke, 2007). Potential sources of these natural preservatives are spices, herbs, fruits, seeds, leaves, barks, and roots (Pratt and Hudson, 1996). *A. danielli* is a spice found in tropical Africa belonging to the genus Aframomum of the family Zingiberaceae. The spice has been reported to exhibit anti-oxidant and preservative properties in different oil and food systems (Adegoke et al., 2003). Antioxidant components of *A. danielli* were identified as phenolic compounds of the trihydroxy type with reducing properties (Adegoke and Gopalakrishna, 1998). Therefore, the effectiveness of *A. danielli* in reducing growth and aflatoxin production. This study was carried out to investigate the effect of local preservative (*A. danielli*) on survival of *A. flavus* in orange juice (acid food) and its ability to produce aflatoxin.

MATERIALS AND METHODS

Matured sweet orange (*C. sinensis*) were obtained at Kuto market in Abeokuta. The oranges were sorted to remove physiologically damaged and blemished ones from the bulk. Dried fruits of *A. danielli* were purchased at Bodija market in Ibadan. The innoculum, a pure culture of *Aspergillus flavus* (reference number IMI3937766) was obtained from Babcock University, Ilisan-Remo, Nigeria. The aflatoxin standard was obtained from 'Romer Labs Inc. (Union, MO, USA)'.

Preparation of orange juice

Orange juice was prepared as described by Bruenmmar (1981). The sweet oranges were washed, peeled and the juice extracted using Kenwood juice extractor. The pulp was sieved to obtain orange juice using muslin cloth and 100 ml each was dispensed into 14 conical flasks. A part of the juice was pasteurized in a water bath at 85°C for 15 min while the other part was unpasteurized. Both samples were kept at room temperature (28±2°C) for 3 and 7 days.

A. danielli treatment and inoculation with *A. flavus*

A. danielli treatment, extraction and incorporation were carried out as described by Jatto and Adegoke (2010). Dry fruits of *A. daniell* were aspirated to release the seeds, winnowed, washed, air-dried and milled into a fine powder. This *A. danielli* powder was used in the preparation of the aqueous extract.

Cold water extraction method was used in preparation of *A. danielli* extract. A concentration of 20% was prepared; 20 g 0f *A. danielli* crude powder was weighed into 100 ml distilled water and the suspension was kept in the refrigerator for 5 days followed by filtration with Whatman no. 1. After the juice preparation, 100 ml of pasteurized and unpasteurized juice samples were transferred into sterile distilled 250 ml conical flask and treated with *A. danielli* extract (1, 2, 3, 4, 5 and 6%). The same quantity of spores suspension of *A. flavus* (6 spores/ml) was added to the samples. No spice was added to the control (0%). The treated samples were covered aseptically and stored at ambient temperature (28±2°C) for 3 and 7 days.

Determination of mycelia dry weight

The *A. flavus* growth was estimated by mycelia dry weight. 50 ml of 3 and 7 day cultures of *A. flavus* in orange juice samples were first inactivated by keeping them for 30 min in 50 ml methanol followed by 50 ml ethyl ether each on a previously weight filter paper (whatman No.1). The mycelia material on the paper was dried at 80°C for 2 h as described by Mayura et al. (1984). Each filter was cooled in desiccators and reweighed until a constant weight was obtained.

Determination of aflatoxin by ELISA method

The AgraQuant Total aflatoxin Assay, a direct competitive enzyme linked immunosorbent assay (ELISA) was used for the estimation of aflatoxin as described by Zheng et al. (2002). The test was performed as a solid phase direct competitive ELISA using horseradish peroxidase conjugate as the competing measurable entity. All reagents and kit components such as standard aflatoxin, enzyme horseradish peroxidise were manufactured by Romer Labs Inc. (Union MO USA). Eight channel pipettes was used to perform the assay which allowed 48 samples to be run at a single experiment. The representative samples were prepared by measuring 20 ml of the sample into 100 ml of 70% methanol in conical flasks, shaken and centrifuged using REML centrifuge at 1500 rpm and then filtered through Whatman no 1. The pH of the samples was adjusted to 7.0 because excessive alkaline or acidity may affect the test results.100 ml of each sample's supernatant and standard (that is, 0, 4, 10, and 20 ppb) were dispensed into 200 ml of conjugate (horseradish peroxidase enzyme in 0.2% BSA at a dilution of 1:10000 and anti-antibody coated well and incubated at room temperature for 15 min. The plate was washed and enzyme substrate was added and allowed to incubate for additional 5 min. Stop solution was then added and the colour change from blue to yellow and read by Biotek ELISA (Elx 800-MS) at 450 nm. The intensity of the resulting yellow colour was measured optically. The results of the optical density of the standards were plotted against standard concentrations. Optical densities of the samples were compared to the optical density of the standards and the interpretation result determined on the graph.

Other measurements

The pH meter (ATC Delta 340) was used for monitoring the pH of

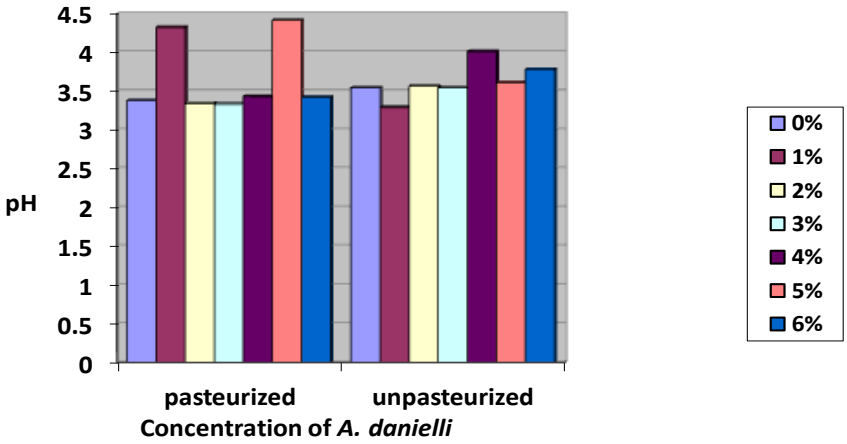

Figure 1. Effect of pH on survival of *A. flavus* and production of aflatoxin in 3 day.

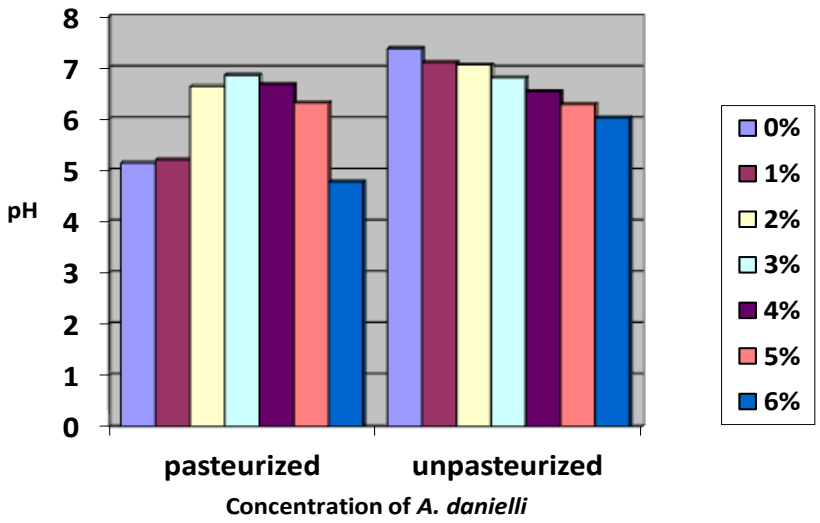

Figure 2. Effect of pH on survival of *A. flavus* and production aflatoxin in 7 day.

the samples. The soluble solid (brix content) was determined using the method described by Uma et al. (2011). The concentration of the sugar was measured directly with a brix refractomer.

Data analysis

Data obtained was analysed using Statistical Package for the Social Sciences (SPSS) version 16. The data were presented as mean and subjected to analysis of variance at 5%.

RESULTS AND DISCUSSION

A decline in pH was observed on day 3 for both pasteurized and unpasteurized samples which ranged between 4.42-3.33 and 4.01-3.29 respectively (Figure 1). This is related to the result obtained by Uma et al. (2011) in a study on determination of shelf life of cashew juice. This drop in pH was more prevalent in unpasteurized juice and may be due to greater rate of fermentation due to presence of other micro-organisms. A tremendous increase in pH was detected on day 7 (6.85-4.77) in pasteurized product and (7.37-6.02) for unpasteurized product (Figure 2).

A factor, which has aided increase in pH, was that most fungi have the capability of secreting extracellular enzymes into their environment in the process of using organic polymers present in the medium as source of carbon and energy and these enzymes secreted may increase the pH (Hussein et al., 1996). The result of mycelia dry weight showed luxuriant growth on pasteurized samples compared to unpasteurized especially at 6 and 0% perhaps due to nutrients supplied by the spice extract when in excess and the inhibition properties of pasteurization which favoured the growth of inoculated spores of *A. flavus* (Figures 3 and 4). The

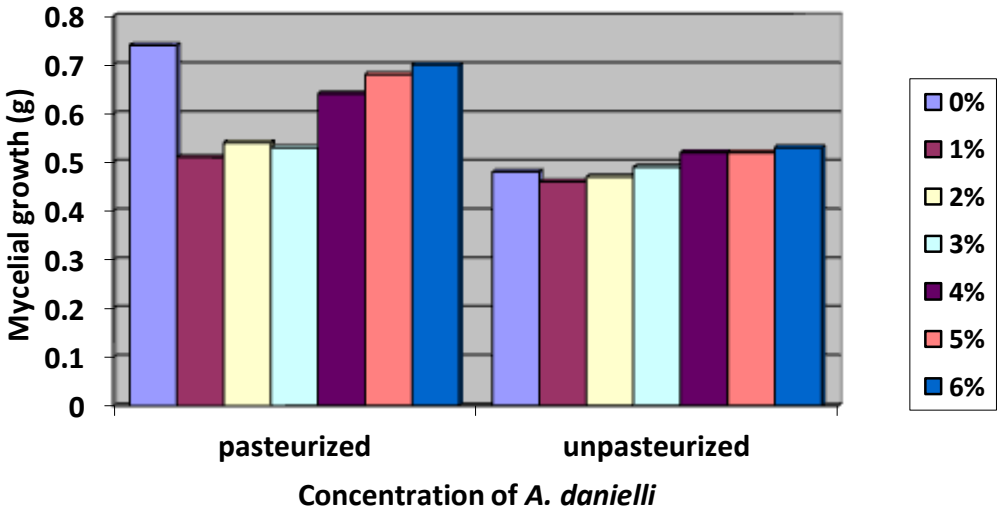

Figure 3. Effect of *A. danielli* on growth of *A. flavus* in orange juice on day 3.

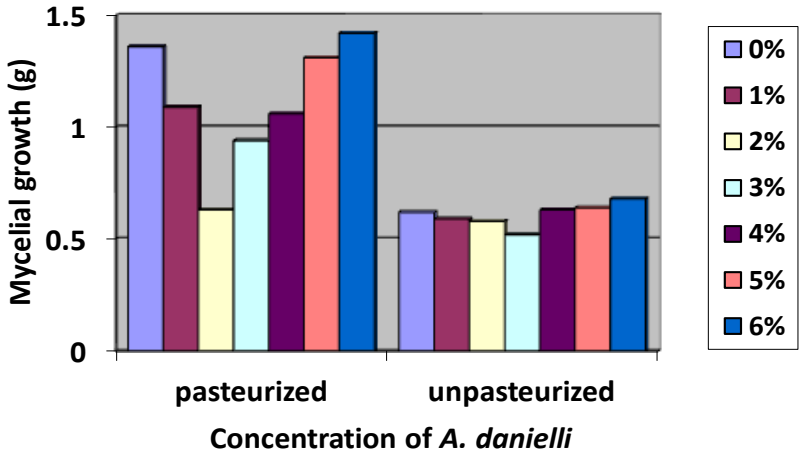

Figure 4. Effect of *A. danielli* on growth of *A. flavus* in orange juice on day 7.

increase in pH might have favoured the growth of *A. flavus* due to the fact that most fungi exhibit good growth over a wide range of pH. A very high growth was observed in 6% concentration of pasteurized sample compare with the control however aflatoxin in the later was higher. Ashaye et al. (2006) reported that some preservative constituents of *A. danielli* might have disrupted the aflatoxin biosynthetic pathway of which resulted to low production in relation to *A. flavus* growth in samples examined. There are no significant difference ($p>0.05$) in growth of *A. flavus* on day 3 for both pasteurized and unpasteurized samples with 1 and 2% *A. danielli* showed minimum growth respectively.

Aflatoxin level did not differ significantly ($p>0.05$) on day 3 for pasteurized and unpasteurized samples and differed significantly ($p<0.05$) on day 7 (Figures 5 to 8). Aflatoxin level was found to be appreciably high in pasteurized samples with the highest value of 156 and 94.50 ng/ml recorded in samples with 0 and 6% respectively. The same trend was observed in unpasteurized samples. Low levels of aflatoxin (below recommended safety limit) were found in pasteurized juice at 2 and 3% concentration on day 3 and 3% concentration on unpasteurized samples while result at 7 day were above recommended safety limits. The effectiveness of low concentration of *A. danielli* extracts is in agreement with the inhibition of the spoilage yeasts and aflatoxigenic moulds by monoterpenes of the spice A. *danielli* extract at low concentration (Adegoke et al., 2000). High levels of aflatoxin recorded with high concentration of extracts may be due to the fact that at higher treatment level, *A. danielli* extracts supplies

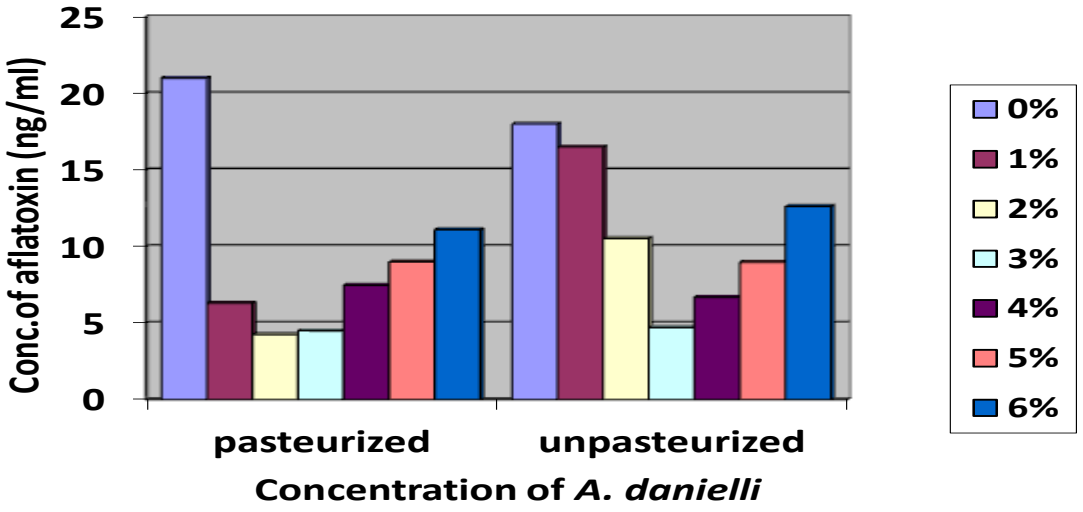

Figure 5. Effect of *A. danielli* on aflatoxin production in orange juice by *A. flavus* on day 3.

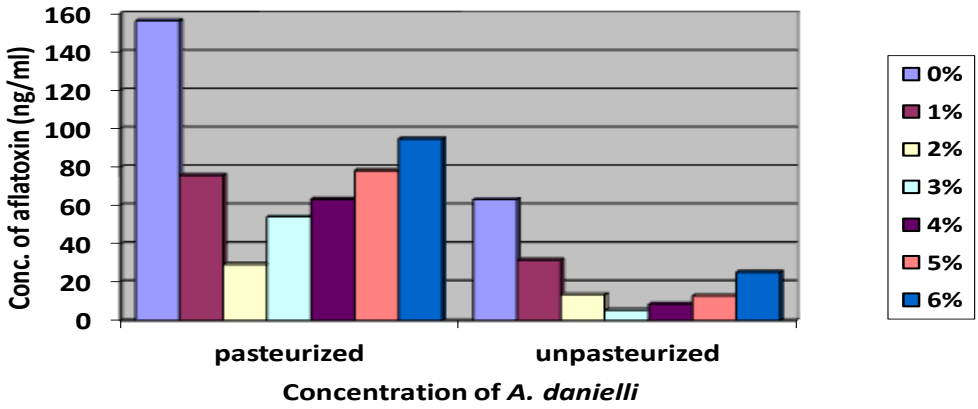

Figure 6. Effect of *A. danielli* on aflatoxin production in orange juice by *A. flavus* on 7 day.

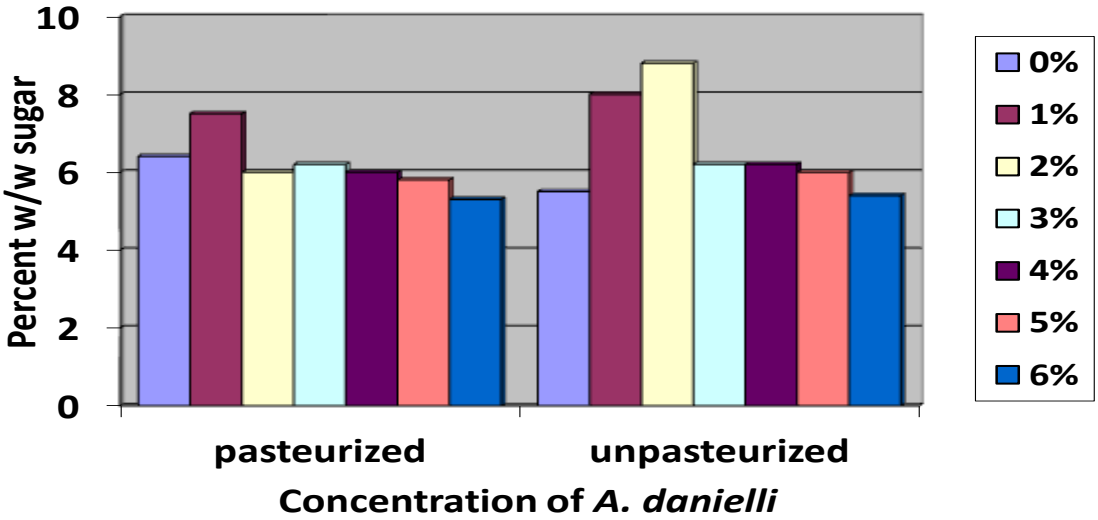

Figure 7. Effect of *A.danielli* on soluble solid of orange juice on day 3.

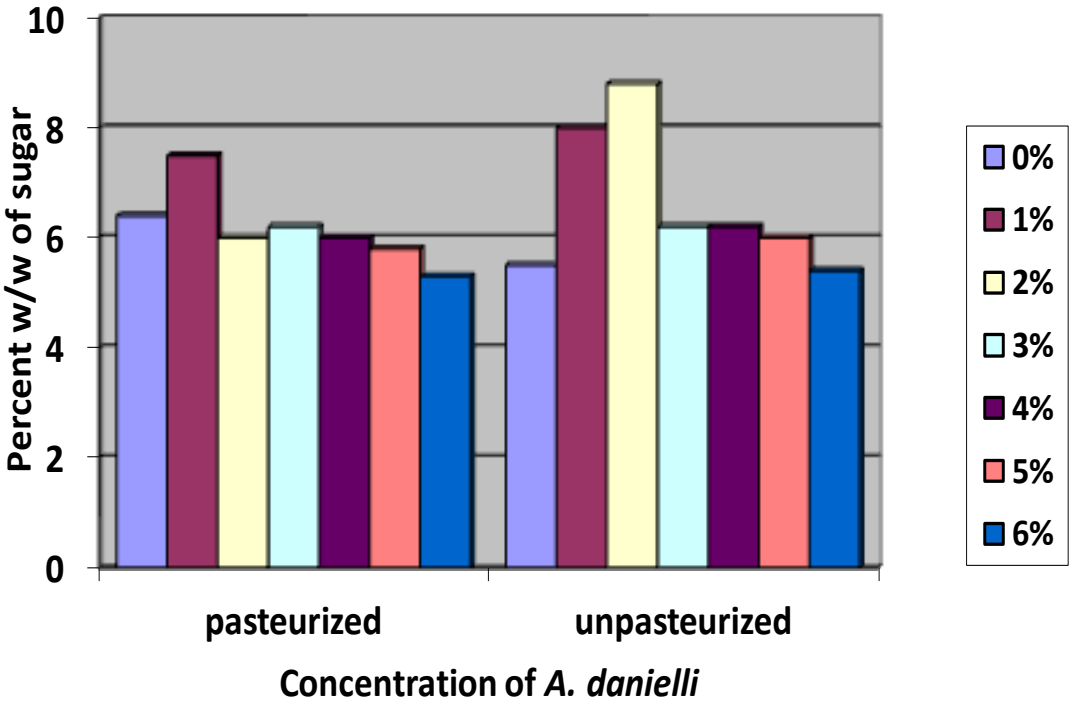

Figure 8. Effect of *A. danielli* on soluble solid of orange juice on day 7.

nutrients for mycotoxigenic mould since the organism can use a wide variety of organic compounds as sources of carbon and energy including carbohydrates, organic acids, aromatic hydrocarbons, which are present in *A. danielli*. The significant level of aflatoxin level of aflatoxin in pasteurized juice found in the study may be probably due to inactivation of other microorganisms during pasteurization process which favoured the growth of *A. flavus*.

The result obtained revealed no difference in brix levels for the treatments (11.00-7.0) and (8.90-7.0) in both pasteurized and unpasteurized orange juice on day 3 but there exist significant difference between the two products at ($p<0.05$). The same trend was recorded (7.50-5.30) and (8.8-5.4) on day 7 of storage. 1 and 2% extracts were favourable for higher retention of soluble sugar (brix) in pasteurized and unpasteurized samples respectively.

Conclusion

The results from the study showed that despite acidity of orange juice, *A. flavus* will survive and produce aflatoxin in orange. *A. danielli* has demonstrated inhibition of *A. flavus* to produce aflatoxin at very low concentration of 2 and 3% for pasteurized and unpasteurized juice respectively with average retention of soluble solid content. The *A. danielli* extract had a higher inhibitory effect in unpasteurised orange juice.

Conflict of Interest

The authors have not declared any conflict of interest.

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