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# Preliminary Investigation of Bio-preservative Effect of *Cola millenii* Extracts on the Shell-Life of "Kunu-Zaki"

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***Authors' contributions***

*This work was carried out in collaboration between the two authors. Author BLA designed the study, wrote the protocol, reviewed the experimental design and all drafts of the manuscript. Author OCA wrote the first draft of the manuscript, handled the laboratory work and performed the statistical analyses. Both authors read and approved the final manuscript.*

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## ABSTRACT

**Aim:** The aim of this study was to investigate the potentials of *Cola millenii* seed and pulp extracts as a possible bio-preservative agent for kunu-zaki, a Nigerian fermented beverage.
**Methodology:** The plant materials were grinded mechanically and macerated in solvent (acetone and ethanol) for 72 hrs. Kunu-zaki was produced using traditional procedures and the reconstituted extracts were aseptically introduced into it. Thereafter, the samples were stored at room temperature and in the refrigerator for 15 days.
**Results:** The pH and total titrable acidity of the freshly produced kunu-zaki were 4.49±0.01 and 0.38±0.00 respectively. The total heterophilic bacterial count of the freshly produced kunu-zaki was 5.6 X $10^2$ cfu/ml, total fungal count was 2.7 X $10^2$ cfu/ml while the lactic acid bacterial count was 11.1 X $10^2$ cfu/ml. Moreover, seven bacteria and six fungi were isolated from the freshly prepared kunu-zaki, they were *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Bacillus licheniformis*, *Micrococcus luteus*, *Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Streptococcus* species, *Geotrichum candidum*, *Saccharomyces cerevisiae*, *Aspergillus niger*, *Rhizopus stolonifer*, *Fusarium* species and

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*Penicillum* species. The seed and pulp extracts of *C. millenii* reduced the bacterial load in the kunu-zaki compared with the controls, and *C. millenii* pulp extracted with ethanol (PEE) totally suppressed microbial growth in kunu-zaki from day 10 onward. *C. millenii* pulp extracted with acetone (PAE) and PEE had the best scores for aroma from day 7 to day 15 of storage while PAE, PEE and kunu-zaki without preservative stored in the refrigerator (FRG) had a significantly higher rating for taste throughout the storage period. The overall acceptability of the bio-preserved products as well as the controls was not significantly different ($p \leq 0.05$) during the first three days and tenth day of storage.
**Conclusion:** From the foregoing, there is great amount of evidence suggesting that the ethanol extract of *Cola millenii* pulp possess bio-preservative potentials against kunu-zaki spoilage. Therefore, it may be expediently exploited in the food and beverage industries to extend the shelf-life of their products.

*Keywords: Bio-peservative; Cola millenii; Kunu-zaki; microbiological; organoleptic.*

## 1. INTRODUCTION

Food spoilage is a metabolic process that causes foods to be undesirable or unacceptable for human consumption due to changes in sensory characteristics and nutritional quality of the food. Spoiled foods may be safe to eat, i.e. they may not cause illness because there are no pathogens or toxins present, but change in texture, smell, taste, or appearance cause them to be rejected [1]. Food loss causes considerable environmental and economic effects. The Food and Agricultural Organizations Research Service estimated that each year, more than ninety-six million tonnes of food are lost by retailers, foodservice and consumers world-wide. Fresh produce and drinks each accounted for nearly 20% of this loss [2]. According to Amusa and Odunbaku [3], shelf life of a food is the time during which it remains stable and retains its desired qualities. Some spoilage are inevitable, and a variety of factors cause deterioration of foods such as endogenous enzymes in plants, microbes (bacteria, molds, yeasts) growing on and metabolizing foods [4]. Microbial spoilage of food is of great concern since food present nearly ideal conditions for the survival and growth of many types of microorganisms. Bacteria and fungi (yeasts and molds) are the principal types of microorganisms that cause food spoilage and food-borne illnesses.

Kunu is a traditional non-alcoholic fermented beverage widely consumed in Nigeria and very popular among Nigerians of all divide. It is usually consumed within few hours of production. Kunu may be contaminated by microorganisms at any time during processing, distribution or handling [3]. The primary sources of microbial contamination are soil, air, water and the processing machinery or utensils.

Previously, artificial chemical preservatives are employed to limit the number of microorganisms capable of growing within foods, but increasing consumer awareness of potential health risks associated with some of these substances, has led researchers to examine the possibility of using natural additives. Plant-derived antimicrobials such as the extracts of herbs and spices are being commonly used in preservation of foods for controlling microorganisms. Presently, the use of natural preservatives in food has provided the basis for the development of new methods of preservation which is bio-preservation or biological preservation of foods. Bio-preservation is the use of natural or controlled antimicrobial as a way of extending the shelf life of food [5]. It aims for reduction of health risks without changing the organoleptic properties of the product [6]. It is a benign ecological approach which is gaining, increasing attention [5]. Consumers and Governments are now moving away from extensive use of artificial preservation [7] which has some negative health implications. These days attention is being turned towards utilization of antimicrobial agents from plants and microorganisms to be used as integral parts of hurdle technology [8]. Using them in combination with other preservative techniques and good manufacturing practice (GMP) can effectively control spoilage bacteria and other pathogens and can inhibit the activities of a wide spectrum of organisms including inherently resistant Gram-negative bacteria [5].

Every culture on earth, through written or oral tradition has relied on the vast variety of natural chemicals found in healing plants for their therapeutic and preservative properties [9]. Such plants can be put into culinary or medicinal use [10]. *Cola millenii* is an example of such plant. It has been reportedly used in the control of vast

range of microorganisms. Earlier studies [11,12] have reported the presence of phytochemicals as well as antimicrobial activities of different parts of the plant. However, there is no report on the utilization of this plant in the control of food spoilage.

## 2. MATERIALS AND METHODS

### 2.1 Collection of Raw Materials for Kunu-zaki Production

The millet (*Pennistum vulgare*) and other ingredients used for the kunu-zaki production were purchased from Sabo market in Akure, they were then transported to the Food Processing Laboratory of Department of Food Science Technology, Rufus Giwa Polytechnic, Owo for further analyses.

### 2.2 Collection and Preparation of Plant Samples

The fruits of monkey kola (*C. millenii*) were harvested fresh from the wild in a forest at Iyere, Owo local government, Ondo state. The plant materials were then authenticated at the Environmental Biology Unit of Department of Science Laboratory Technology, Rufus Giwa Polytechnic, Owo and voucher specimen (CMF112IX-*C.millenii* fruit) was deposited at the Department of Forestry Resources Technology, of the same institution. The seed and pulp were removed from the fruit manually using laboratory knife. Thereafter, they were rinsed thoroughly in distilled water and air dried for three weeks in the laboratory. The dried samples were then ground into powder with the aid of a mechanical grinder and were stored in clean air- tight containers, and kept in a cool, dry place until required for use.

### 2.3 Reagents and Chemicals

All reagents and chemicals were of analytical grade and were obtained from the Department of Science Laboratory Technology, Rufus Giwa Polytechnic, Owo, Ondo State Nigeria.

### 2.4 Experimental Design

#### 2.4.1 Extraction procedure

One hundred gram (100 g) portion of the powdered sample was soaked in 300 ml of different solvents (acetone and ethanol) for 48 hrs with intermittent stirring using sterile spatula. The plant extracts were then filtered through Whatman No 1. filter paper into McCartney bottles and then dried using rotary evaporator at a temperature of 50℃ to yield crude extracts [13]. Different concentrations of the extracts were prepared by diluting 1.0 g of the extracts in 100 ml of 0.01% Tween-20 to obtain concentrations of 100 mg/ml [14].

#### 2.4.2 Production of Kunu-zaki samples

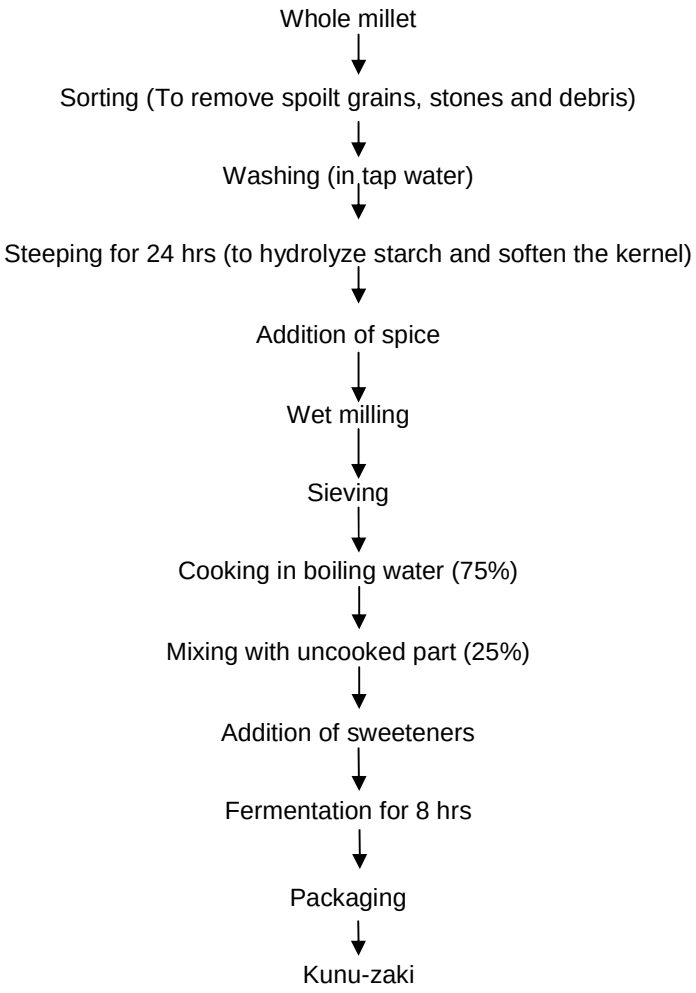
Two hundred and fifty grams (250 g) of millet was steeped in 500 ml of distilled water in an airtight plastic container for 24 hrs (primary fermentation). Thereafter, the grain was washed and mixed with spices (ginger 35 g, clove 7.5 g, African black pepper 7.5 g); the mixture was wet-milled and sieved. The resultant slurry was divided into two parts (3:1). The larger part was cooked by adding boiling water (100℃) and allowed to cool to about 50℃ after which the uncooked part was added to the cool cooked part and then homogenized. Sweeteners were added to taste during the mixing, and then the mixture was allowed to ferment (secondary fermentation) for 8 hrs after which it was packaged (see Flowchart 1).

#### 2.4.3 Bio-preservation protocol

Just before packaging, the kunu-zaki was divided into various parts thus:

Sample 1 = 75 cl Kunu-zaki + 100 mg/ml acetone extract of *C. millenii* seed thereafter referred to as SAE
Sample 2 = 75 cl Kunu-zaki + 100 mg/ml ethanol extract of *C. millenii* seed thereafter referred to as SEE
Sample 3 = 75 cl Kunu-zaki +100 mg/ml acetone extract of *C. millenii* pulp, thereafter referred to as PAE
Sample 4 =75 cl Kunu-zaki +100 mg/ml ethanol extract of *C. millenii* pulp, thereafter referred to as PEE
Sample 5 = 75 cl Kunu-zaki without preservative and kept in the fridge thereafter referred to as FRG
Sample 6 = 75cl Kunu-zaki without preservative and kept under room temperature thereafter referred to as CNT.

All samples were stored and monitored for 15 days.




**Flowchart 1. For the production of kunu-zaki**

### 2.4.4 Microbiological analysis

All the samples including the controls (those without extracts) were subjected to microbiological analysis at regular intervals using serial dilution and pour plated in triplicates on the following media (1) Nutrient agar for estimation of bacteria, (2) MRS agar for the enumeration of lactic acid bacteria, (3) PDA agar for estimation of fungi, these were prepared using manufacturer's specifications and incubated at their respective optimum temperatures and duration. Bacterial isolates were identified using the method of Ojokoh and Oyetayo [15] while the plate morphological characteristics of the fungi were cross-referenced with the features described by Barnet and Hunter [16].

### 2.4.5 pH and total titrable acidity assay

A mixture of 10 ml of each sample was used for pH determination as described in Association of Analytical Chemists [17]. Total titratable acidity (TTA) was determined by titrating 20 ml of the same sample against 0.1 M NaOH.

### 2.4.6 Organoleptic evaluation

This was done by 20 trained panelists selected randomly from Department of Food Science Technology, Rufus Giwa Polytechnic, Owo Ondo state. The nine point hedonic scale was used (score "9" having excellent attribute and Score "1" indicating dislike extremely). Samples were coded with random alphabets. The properties evaluated were appearance, taste, aroma and overall acceptability.

## 2.5 Statistical Analysis

Unless otherwise indicated results are expressed as means ± SD of three replicates. Data were subjected to one –way analysis of Variance (ANOVA) using SPSS version 16.0. The Duncan's Multiple Range test was used to separate the means at the 5% level of probability.

## 3. RESULTS AND DISCUSSION

### 3.1 pH and Titrable Acidity of Freshly Produced Kunu-zaki

The pH and total titrable acidity of the freshly produced kunu-zaki were 4.49±0.01 and 0.38±0.00 respectively (Table 1). These values are within the range reported for fermented cereal products [18,19]; the low pH and acidity observed may be linked with acid production by some bacteria during fermentation which involves the degradation of carbohydrates [20].

**Table 1. pH and total titratable acidity of freshly produced kunu-zaki**

| Parameter | Value |
|---|---|
| pH | 4.49±0.01 |
| TTA | 0.38±0.00 |

### 3.2 Microbiological Quality of Freshly Produced Kunu-zaki

The total heterophilic bacterial count of the freshly produced kunu-zaki was 5.6 X $10^2$ cfu/ml, total fungal count was 2.7 X $10^2$ sfu/ml while the lactic acid bacterial count was 11.1 X $10^2$ cfu/ml, these results are in agreement with the observation of Akoma et al. [19]. A total of seven (7) bacteria and six (6) fungi were isolated from the freshly prepared kunu-zaki, they were *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Bacillus licheniformis*, *Micrococcus luteus*, *Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Streptococcus* species, *Geotrichum candidum*, *Saccharomyces cerevisiae*, *Aspergillus niger*, *Rhizopus stolonifer*, *Fusarium* species and *Penicillum* species. Most of these organisms have been reported by Lichtenwalner et al. [21], Olasupo et al. [22] and Ugwuanyi et al. [23] to take part in fermentation of various types of cereal products especially kunu-zaki and ogi. And they may have been introduced into the product from the cold uncooked part during the production of the product. These organisms if not removed after production may continue to ferment the product leading to total spoilage of the product as observed by [23].

### 3.3 Effect of *C. millenii* Extracts on the Microbiological Quality of Stored Kunu-zaki

Immediately after production, just before packaging the product was divided into six (6) parts and different extracts of *Cola millenii* were added as bio-preservative and are well labeled. Therefore, the following codes are used for the purpose of this discussion; SAE= 75 cl Kunu-zaki + 100 mg/ml (5 ml) acetone extract of *C. millenii* seed, SEE= 75 cl Kunu-zaki + 100 mg/ml (5ml) ethanol extract of *C. millenii* seed, PAE= 75 cl Kunu-zaki +100 mg/ml (5 ml) acetone extract of *C. millenii* pulp, PEE= 75 cl Kunu-zaki +100 mg/ml (5 ml) ethanol extract of *C. millenii* pulp, FRG= 75 cl Kunu-zaki without preservative and kept in the refrigerator; and CNT= 75 cl Kunu-zaki without preservative and kept under room temperature.

The assessment of total heterophilic bacteria count in the bio-preserved kunu-zaki revealed that the seed and pulp extracts of *C. millenii* reduced the bacterial load in the kunu-zaki compared with the controls (Figs. 1- 3). There was no observed growth on the nutrient agar and potatoe dextrose agar plates inoculated with

**Table 2. Microbial quality of freshly produced kunu-zaki**

| Type of organisms | Count x$10^2$ cfu/ml | Isolates |
|---|---|---|
| Total heterotrophic bacteria | 5.6 | *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Bacillus licheniformis*, *Micrococcus luteus* |
| Lactic acid bacteria | 11.1 | *Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Streptococcus* species |
| Total fungal count | 2.7 | *Geotrichum candidum*, *Saccharomyces cerevisiae*, *Aspergillus niger*, *Rhizopus stolonifer*, *Fusarium* species, *Penicillum* species |

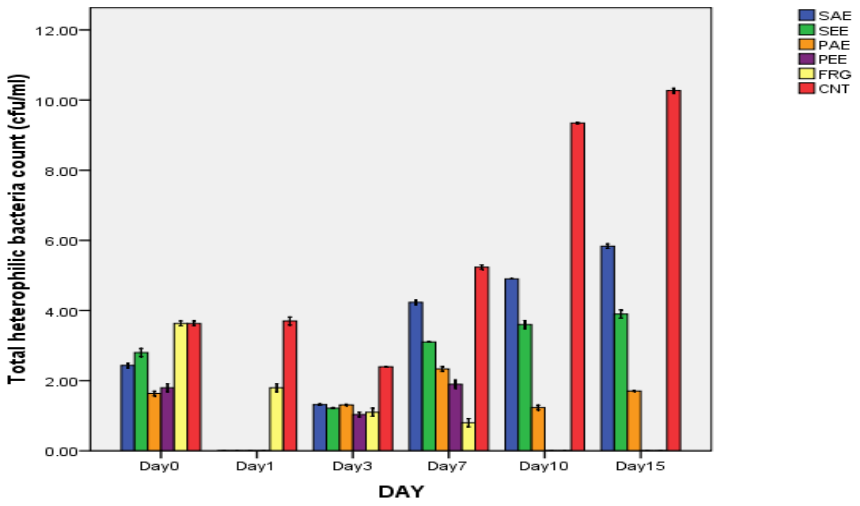



**Fig. 1. Total heterophilic bacteria count on bio-preserved kunu-zaki during storage (x10$^2$ cfu/ml)**

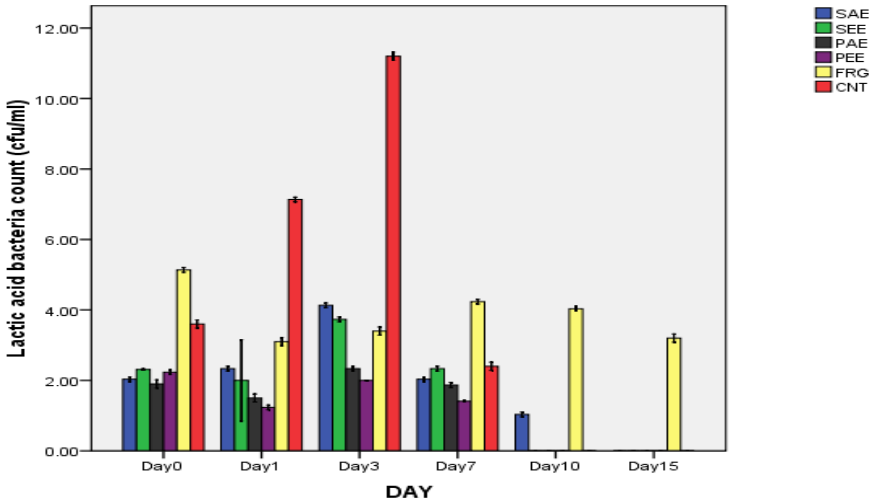



**Fig. 2. Lactic acid bacteria count on bio-preserved kunu-zaki during storage (x10$^2$ cfu/ml)**

preserved samples on day 1, this suggests that the *C. millenii* extracts may possess antimicrobial activities which suppressed the growth of these organisms. Also, there was a reduction in the lactic acid bacteria count on day 1 which signified that the extracts inhibited their growth. However, there were microbial growths in all the samples on day 3 which increased on day 7 but showed gradual decline from day 10 to 15. It is interesting to know that PEE totally suppressed the microbial growth in the product from day 10 onward while there were still growths in other samples including the refrigerated sample. Also, the lactic acid bacteria disappeared in all the samples on day 10 except SAE and RFG but fungi persisted in all the samples throughout the storage period except in PEE from which fungi disappeared on day 15. The persistence of lactic acid bacteria in the RFG has been reported by earlier investigators in probiotic foods [20,21]. The reduction in the microbial growth in the bio-preserved samples and especially the total disappearance of the microorganisms from PEE on day 15 indicates that the ethanol extract of *C. millenii* pulp may contain antimicrobial principles which may be exploited in food preservation. This corroborates the reports of earlier researchers who reported the antimicrobial activities of *Cola millenii* extracts especially the seed and leaf [22].

**Table 3. Organoleptic properties of freshly produced kunu-zaki**

| Parameter | Value |
|---|---|
| Appearance | 8.01±0.01 |
| Aroma | 7.12±0.04 |
| Taste | 8.2±0.00 |
| Overall acceptability | 7.87±0.01 |

*Key: Each data is the mean± standard error of 20 member taste panelist (9-point hedonic scale: 9= Excellent, 7=like extremely, 6= like very much, 5= like slightly, 4= neither like nor dislike, 3= dislike slightly, 2= dislike very much, 1= dislike extremely)*

### 3.4 Effect of *C. millenii* Extracts on the Organoleptic Properties of Stored Kunu-zaki

The organoleptic characteristics of the bio-preserved kunu-zaki during storage period is presented in Figs. 4 to 7. The results showed that the quality of the appearance of all the samples were not significantly different ($p \leq 0.05$) after day 1 of storage but there was a sharp decline in the quality of the appearance of the bio-preserved samples with scores $6.62 \pm 0.05^{b}$, $5.85 \pm 0.01^{c}$, $5.06 \pm 0.02^{d}$, $4.03 \pm 0.00^{e}$ for SAE, SEE, PAE and PEE respectively on day 3 compared with the controls ($7.00 \pm 0.02^{a}$ and $6.96 \pm 0.01^{a}$ respectively for FRG and CNT). The appearance of PAE and PEE from day 7 till the end of the experiment scored 1 which represent extremely poor. This is probably due to the change observed in the colour of the PAE and PEE from ash-white to light brown. This change suggests that the pulp extracts may contain some chemical species which reacted with the beverage milieu to produce the change in colouration. This has not been reported earlier and there is a need to investigate further into the cause of the change in coloration.

On the basis of aroma, there was no significant differences between the quality of the aroma of all the samples in the first three days of storage the samples PAE and PEE had the best scores for aroma from day 7 to day 15 of storage while samples SAE, SEE and CNT had the least scores respectively.

From taste view point, the samples PAE, PEE and FRG had a significantly higher scores compared with the others from day 3 to day 15 of the storage. Only sample FRG had a better score ($6.15 \pm 0.02^{a}$) than PAE and PEE with scores of $4.83 \pm 0.00^{b}$ and $5.12 \pm 0.01^{b}$ respectively.

The overall acceptability of the bio-preserved products as well as the controls was not significantly different ($p \leq 0.05$) during the first three days of storage. This is in consonance with the report of Ugwuanyi et al. [23]. However, the acceptability of the PAE and SEE were significantly lower than the others from day 7 to 15. This may be due to the appearance of the samples. In all, FRG sample had the best overall acceptability throughout the experimental period and could be traced to the stability effect of the low storage temperature on the biochemical changes in the kunu-zaki milieu.

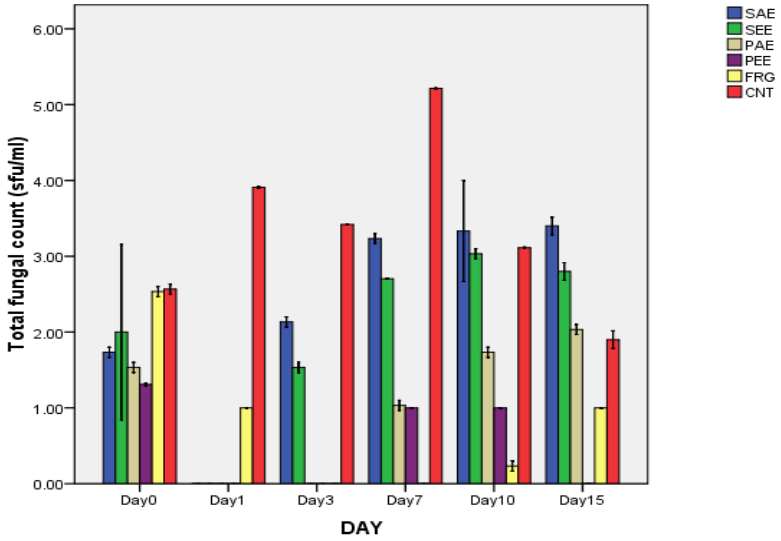



**Fig. 3. Total fungal count on bio-preserved kunu-zaki during storage ($x10^2$ sfu/ml)**

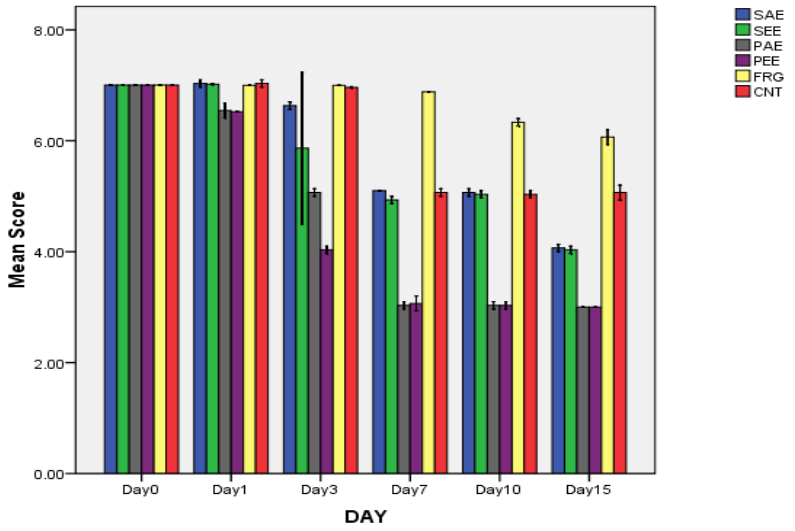



**Fig. 4. Assessment of the appearance of bio-preserved kunu-zaki during storage**

*Key: Each data is the mean± standard error of 20 member taste panelist (9-point hedonic scale: 9= Excellent, 7=like extremely, 6= like very much, 5= like slightly, 4= neither like nor dislike, 3= dislike slightly, 2= dislike very much, 1= dislike extremely), SAE= 75 cl Kunu-zaki + 100 mg/ml acetone extract of C. millenii seed, SEE= 75 cl Kunu-zaki + 100 mg/ml ethanol extract of C. millenii seed, PAE= 75 cl Kunu-zaki +100 mg/ml acetone extract of C. millenii pulp, PEE= 75 cl Kunu-zaki +100 mg/ml ethanol extract of C. millenii pulp, FRG= 75 cl Kunu-zaki without preservative and kept in the fridge; and CNT= 75 cl Kunu-zaki without preservative and kept under room temperature*

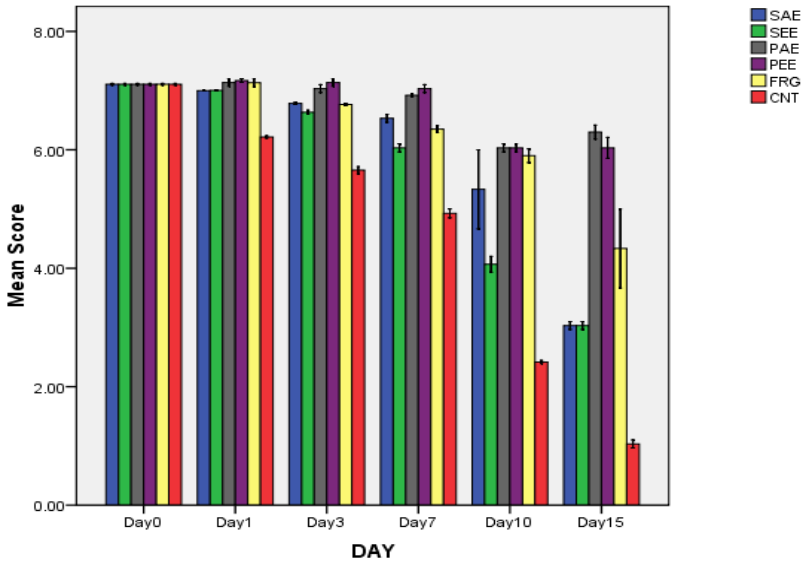



**Fig. 5. Assessment of the aroma of bio-preserved kunu-zaki during storage**

*Key: Each data is the mean± standard error of 20 member taste panelist (9-point hedonic scale: 9= Excellent, 7=like extremely, 6= like very much, 5= like slightly, 4= neither like nor dislike, 3= dislike slightly, 2= dislike very much, 1= dislike extremely), SAE= 75 cl Kunu-zaki + 100 mg/ml acetone extract of C. millenii seed, SEE= 75 cl Kunu-zaki + 100 mg/ml ethanol extract of C. millenii seed, PAE= 75 cl Kunu-zaki +100 mg/ml acetone extract of C. millenii pulp, PEE= 75 cl Kunu-zaki +100 mg/ml ethanol extract of C. millenii pulp, FRG= 75 cl Kunu-zaki without preservative and kept in the fridge; and CNT= 75 cl Kunu-zaki without preservative and kept under room temperature*

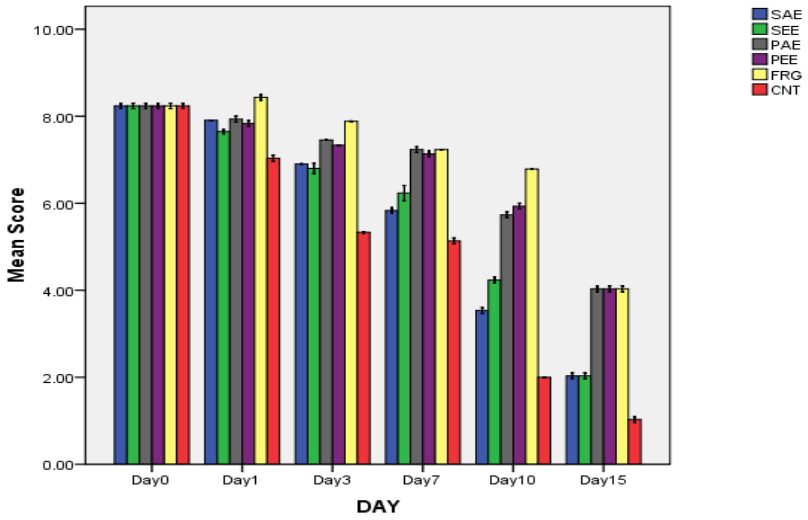



**Fig. 6. Assessment of the taste of bio-preserved kunu-zaki during storage**

*Key: Each data is the mean± standard error of 20 member taste panelist (9-point hedonic scale: 9= Excellent, 7=like extremely, 6= like very much, 5= like slightly, 4= neither like nor dislike, 3= dislike slightly, 2= dislike very much, 1= dislike extremely), SAE= 75 cl Kunu-zaki + 100 mg/ml acetone extract of C. millenii seed, SEE= 75 cl Kunu-zaki + 100 mg/ml ethanol extract of C. millenii seed, PAE= 75 cl Kunu-zaki +100 mg/ml acetone extract of C. millenii pulp, PEE= 75 cl Kunu-zaki +100 mg/ml ethanol extract of C. millenii pulp, FRG= 75 cl Kunu-zaki without preservative and kept in the fridge; and CNT= 75 cl Kunu-zaki without preservative and kept under room temperature*

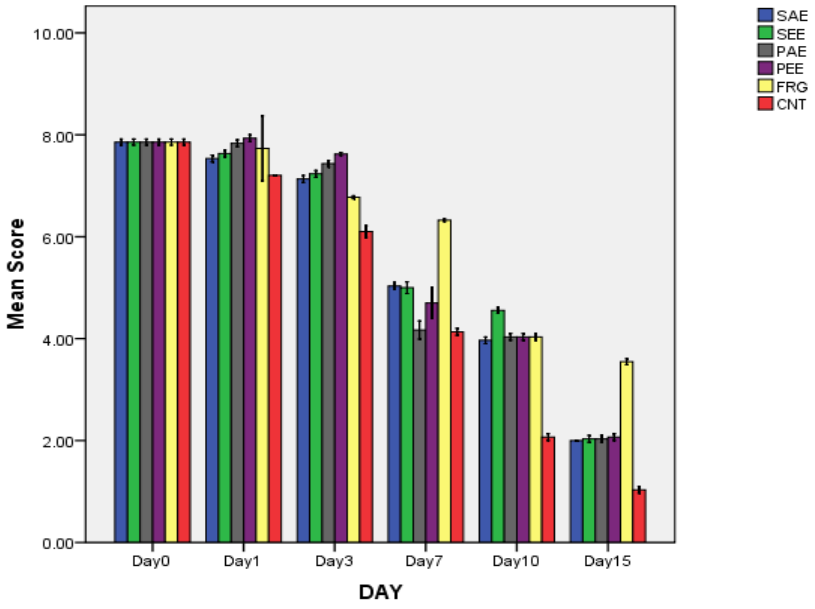



**Fig. 7. Assessment of the overall acceptability of bio-preserved kunu-zaki during storage**

*Key: Each data is the mean± standard error of 20 member taste panelist (9-point hedonic scale: 9= Excellent, 7=like extremely, 6= like very much, 5= like slightly, 4= neither like nor dislike, 3= dislike slightly, 2= dislike very much, 1= dislike extremely), SAE= 75 cl Kunu-zaki + 100 mg/ml acetone extract of C. millenii seed, SEE= 75 cl Kunu-zaki + 100 mg/ml ethanol extract of C. millenii seed, PAE= 75 cl Kunu-zaki +100 mg/ml acetone extract of C. millenii pulp, PEE= 75 cl Kunu-zaki +100mg/ml ethanol extract of C. millenii pulp, FRG= 75 cl Kunu-zaki without preservative and kept in the fridge; and CNT= 75 cl Kunu-zaki without preservative and kept under room temperature*

## 4. CONCLUSION

From the foregoing, there are encouraging indices suggesting that the ethanol extract of *Cola millenii* pulp may possess bio-preservative potentials of kunu-zaki as evidenced in the microbiological and organoleptic properties of the bio-preserved and stored products. Therefore, it may be expediently exploited in the food and beverage industries to extend the shelf-life of their products. However, there is need to determine the toxicity properties of the plant material and further work on removing the colour changing effect of the pulp in kunu-zaki

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## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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