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Evaluation of the Microbial Flora and Shelf-Life Stability of Two Indigenous Palm Wine Products (*Elaies guineensis* and *Raphia hookeri*) Obtained from Southwest Nigeria

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A B S T R A C T

The microbiological and biochemical changes as well as the shelf-life of *Elaeis guineensis* and *Raphia hookeri* types of palm wines were determined for 5 days in a laboratory experiment. *E. guineensis* brand was found to harbour less heterotrophic and coliform bacteria population than the *R.hookeri*. Whereas, the latter harboured more yeast species. *R. hookeri* was found to be richer in nutrients than *E. guineensis.* Microbiological characterization of isolates following standard and conventional methods revealed the presence of *Bacillus sp., Lactobacillus sp., Brevibacterium sp.* and *Staphylococcus sp.* from *E. guineensis* while *Escherichia coli* and *Micrococcus* species *were* additional isolates obtained from *R. hookeri. Saccharomyces and non-saccharomyces yeasts were isolated from both palm wine types.* Furthermore, heterotrophic count and pH values were observed to decrease with increased fermentation days. The bio-preservative effects of leaves and stem bark of *Vernonia amygdalina, Nauclea vandeguchuti* (opepeira) and *Euphobia* sp. respectively on two palm wine types namely, *Elaeis guineensis* and *Raphia hookeri* from IlogboEremi and Ado-Ado/Ota, Nigeria was evaluated. The effect of plant extracts used as preservatives on the isolates from the palm wine samples were determined. The combination of all the preservatives *Vernonia amygdalina, Nauclea vandegucluti* and *Euphorbia* sp. lowered the bacteria and fungi load compared to those of the control sample, the individual plant preservatives kept the pH fairly constant in the samples. *R. hookeri* samples had more crude protein, fat and vitamin E while *E. guineensis* had less crude protein and fat.

Introduction

Palm wine is an alcoholic beverage obtained from the fermented sap of various palm trees; it can be collected and tapped from the palm tree – *Elaies guineensis* or from the raffia tree – *Raphia hookeri*. Thereafter, sorted and fermentation begins immediately after collection as a result of activity of natural yeasts in the air, acting on the sugar. The alcohol concentration reaches approximately 4% within two hours. Although, at this stage, the product is a sweet, white, milky intoxicating aromatic beverage. Fermentation continues for up to 24hours resulting in a more alcoholic, acidic and sour white drink (Mbuagbaw *et al.,* 2012). Palm wine is consumed throughout the tropics and appears as a whitish liquid produced by natural fermentation of the sap of *Elaeis guineensis* and *Raphia hookeri* (Uzochukwu *et al.*, 1991). The unfermented sap is clean, sweet, colourless syrup which contains 10-20% of sugar which is mainly sucrose (Okafor, 1975a),it also contains nutritionally important components such as amino acids, proteins, P, Mg, Zn and vitamins upon fermentation by the natural microbial flora (Okafor, 1978; Ezeagu *et al.,* 2003). The sugar level decrease rapidly as it is converted to alcohol and other products (Obire, 2005) whereas the sap becomes milky white due to the increased microbial suspension in it (Okafor, 1975 a,b).

The sap of the palm trees, which is originally *sweet* (Atputharajah *et al.,* 1986; Amoa-Awua *et al.,* 2007; Naknean *et al.,* 2010; Santiago-Urbina *et al.,* 2013) serves as a rich substrate for the growth of various types of microorganisms. The sap undergoes spontaneous fermentation, which promotes the proliferation of yeasts and bacteria for the conversion of the sweet substance into several metabolites mainly ethanol, acetic acid, Lactic acid, (Amoa-Awua *et al.,* 2007; Stringini *et al.,* 2009; Ouoba *et al.,* 2012; Santiago-Urbina *et al.,* 2013). Palm sap fermentation has been reported to be an alcoholic, lactic and acetic fermentation (Okafor, 1977; Atputharajah *et al.,* 1986; Amoa-Awua *et al.,* 2007; Stringini, *et al.,* 2009; Ouoba *et al.,* 2012; Santiago—Urbina *et al.,* 2013). Therefore, yeast, lactic acid bacteria and acetic acid bacteria are considered to play the most important role in palm wine production.

The palm sap is obtained through the process known as tapping, which involves a series of operations to stimulate the flow of sap (Atputharajah *et al.,* 1986), such as the perforation of the trunk, insertion of a tube in the hole and collection of the sap in a container (gourd, clay pot, plastic container, glass bottle or calabash) (Ouoba *et al.,* 2012). There are diverse ways of tapping palm trees, which depends on the locality, but in general two methods are practiced. In the first method; the sap is obtained from a live standing tree, this process implies climbing very tall palm trees, and perforation of the trunk in the top of the tree (Ouoba *et al.,* 2012), or cutting into the end of spadix from the tender inflorescence of the palm tree (inflorescence tapping). In the second method; the tree is felled or cut down before tapping (Stem tapping), such as palm wine from Ghana and ‘’Taberna’’ production in Mexico. The cessation of the flow of palm sap varies according to the palm tree species and from tree to tree; for instance, the shorter duration of tapping could be two weeks (2 weeks) and the longest duration of tapping 8 weeks (Balick, 1990; Amoa – Awua*et al.,* 2007; Santiago-Urbina *et al.,* 2013). Palm wine is collected twice a day, normally in the morning and the evening, it can be either immediately consumed or stored for later sale (Amoa-Awua *et al.,* 2007; Naknean *et al.,* 2010; Santiago-Urbina *et al.,* 2013).

Palm wine is characterized by an effervescence of gas resulting from the

fermentation of the sucrose content (Bassir, 1962), by the fermenting organisms. Previous studies on the microbiology of *E. guineensis* and *R. hookeri* revealed several bacterial and yeast florae to be involved in the fermentation process (Faparusi, 1987). These organisms have been reported to originate from several sources which include tapping equipment, containers and the environment (Faparusi and Bassir, 1972a).

Fermentation is a metabolic conversion of carbohydrate such as sugar into an alcohol or an acid using yeast, bacteria or a combination thereof (mixed culture). It is also the slow decomposition of organic substance of plant and animal origin which is enzyme-mediated. In this process also starch is broken down into fermentable sugars by fungal enzymes such as alpha amylase to facilitate fermentation by yeasts which includes many *Saccharomyces species*. Fermentation could occur under anaerobic or aerobic conditions and yield lactate, acetic acid, ethanol, carbon dioxide or some other simple product (Adams and Nout, 2001). However, there are numerous important products obtained during palm wine fermentation such as antibiotics, vitamins, food supplements and blood plasma expanders (Amoa-Awua *et al.,* 2007)

Palm wine differs from conventional beers and table wines produced in the modern brewery and winery in three ways; firstly, the substrate fermented for such beers are usually grains while for the wines; grapes and fruit juice are used in wine. The basic principle is however, the same. Sugar solution is fermented, essentially by yeast (*Saccharomyces sp.*). Secondly, whereas there is a fermentation control during the production of modern beers and wines. Fermentation of palm wine is not controlled. Thirdly, the European beer and wines are usually clarified by removing microbial cells and other suspended material. Palm wine comparatively is consumed without such clarifications. In essence, the basic differences between the true wines and the palm wine are a matter of technological difference between wine - making techniques and palm wine methods. The methods of producing palm wine are likely to continue to change with enhanced technological advancement of the consuming countries (Orimaiye, 1997; Ezeronye, 2004).

Theodore *et al.,* (2020) reported that Palm wine is one of the most widely consumed alcoholic beverage in West Africa, yet it is constituted of advanced micro-biota and metabolites, which offers its consumers the unique quality and value-addition. They studied the genome of the microbiota of fermented saps taken from at least three palm tree species in Cote Ivoire. Their work gave useful understanding into microbiology and biochemistry of palm wines as well as the basis for hand - picking microorganisms of relevance in industrial production of alcoholic beverage. Coutino*et al.,* (2020) made a similar report using the Mexican local beverage made from fermented sap coyol palm (*Acromia aculeata*). The Ethiopian fermented alcoholic beverage was analysed and found to have similar microbiota (Lemi, 2020). The process of understanding Yeasts ecology, discovery of non-Saccharomyces yeasts and genomic aggregation is pivotal to improvement of quality of wines, engagement of high-throughput technology by sequencing yeasts genes that would consequently improve fermentation as well as the quality of alcoholic beverage has been suggested (Weina *et al.,* 2020; Guaragnella *et al.,* 2020; Adebowale *et al.,* 2020).

This study is therefore aimed at evaluating the microbiological flora of the two brands of indigenous palm wine, the biochemical changes associated with the sap fermentation and the effect of application of traditional

plant preservatives on shelf – life of the products.

Materials and Methods

Sample collection

Fresh palm wine samples from oil palm tree (*E. guineensis*) and Raphia palm (*R. hookeri*) were separately collected from traditional palm wine tappers from Badagry (Ilogbo Eremi) Lagos State and Ado-Odo/Ota Ogun - State, Nigeria. The freshly tapped samples were collected using 10 pre – sterilized labelled 100ml capacity sample bottles with perforated screw caps. The perforated screw caps were plugged with sterile non-absorbent cotton wool.

Plant materials

The leaves and stem barks of *Vernonia amygdalina, Nauclea vandegucluti* (Opepeira) and *Euphorbia* sp. respectively were collected from Traditional medicinal herb seller (Eleweomo) at IlogboEremi, Badagry, Lagos State, Nigeria. The plant materials were collected and washed with sterilized water and then with absolute ethanol and air- dried (at room temperature for 14 days). The dried leaves and stem bark were then ground to powder in a mechanical blender (Philips, USA) and mixed together.A total of 12 bottles, 6 for each of the two palm wine samples, were labelled thus:

Control (C): Sample without preservative
T0: Sample with only *Varnonia amyadalina*
T1: Sample with only *Nauclea sp.* stem back
T2: Sample with only *Nauclea sp*. leave
T3: Sample with only *Euphorbia sp*. Leave
T4: Sample of all plant materials

Preservation treatments

Approximately, Five 40 ml sample of each of *Raphia* and *E. guineensis* palm wines were treated with a total of 5 types of preservative namely, *Vernonia amygdalina* only (TO) *Nauclea sp.* only (T1), (Stem barks), *Nauclea sp. (leave)* only (T2) *Euphorbia* sp (leave) only (T3) and combination of (T0), *Vernonia amygdalina* only, *Nauclea sp.* stem bark (T1), *Nauclea sp.* leave T2, *Euphorbia sp* leave T3.However, one sample bottle was left without any form of preservative (C) as control. The treatment was carried out by adding 10mg of each powdered traditional preservative to the sterile sample bottles, but 5mg each of all plant materials for T4. Thereafter, 40mls of fresh palm wine sap were added, gently shaken to mix and allowed to stand in a laboratory glass cabin sterilized using 2.2% acid alcohol (Njoku *et al.*, 1990).

Media

Seventy two grams (72g) of Baird parker (BPA) agar, twenty-three grams (23g) of nutrient agar, fifty-two grams (52g) of MacConkey agar and thirty-nine grams (39g) of Potato dextrose agar were weighed using a digital metre balance (sutorium) and were suspended into 1- litre (1000ml) amount of distilled water respectively, homogenized on hot-plate magnetic stirrer to form a uniform solution.

Isolation of microbial flora using serial dilution technique

Standard and conventional methods were followed in isolating the microbial flora (Pearson, 1999).

Microbial isolation

Approximately, 1-ml aliquots of each palm wine sample were taken aseptically at 0, 24,

48, 72, 96 and 120 hours of fermentation and were serially diluted 10 - fold in 0.1% (w/v) bacteriological peptone. Thereafter, 1 ml dilutions were inoculated in duplicates using total spread plate method on tryptone SO_4 agar (Oxoid) for total heterotrophic bacterial count, MacConkey agar (Oxoid) for the total coliform count and Sabouraud dextrose agar (Oxoid) containing 0.05mg/ml chloramphenicol for yeast counts, described by Cruickshank *et al.,* (1982) and Ojomo *et al.,* (1984).

Characterization and identification of bacterial isolates

Pure cultures of bacterial isolates from preserved palm wine and non- preserved were identified based on their colonial morphology, cellular morphology and biochemical characteristics. The organisms were subsequently characterized according to the taxonomic scheme of Buchanan and Gibbons (1994).

Biochemical characterization of bacterial isolates

Standard and conventional methods were engaged in the characterization of isolates which include; Carbohydrate utilization test, starch hydrolysis test, Gram reaction test, Gelatin hydrolysis test, Methyl-red Voges Proskauer test, Urease test, Coagulase test, Nitrate reduction test, Indole test and many others (Cowan and Steel, 1990; Collins *et al.,* 1989).

Isolation procedures for yeast

The medium used for isolation was yeast extract peptone dextrose Agar medium (YEPDA) / Malt Extract Agar (MEA). The composition of the medium in g/l: Yeast extract 15g, peptone 20g, dextrose 20g, agar 20g. In order to inhibit bacterial growth, streptomycin sulphate (0.14g/l) was added to the autoclaved medium. Isolations were made from the fermented palm wine using the methods of Barnett *et al.,* (1990) and Kregger van- Rij (1987). Inocula were obtained from these slants for successive studies.

Identification of yeast isolates morphological studies

Colonies developing on plate were group on the basis of their colonial morphology. Cultural features examined were the elevation of colonies, shape, degree of growth, colour, edge and opacity. Approximately, 2.0g of malt extract broth powder and 20g agar-agar were weighed and dissolve in 1 litre of distilled water. The solidified media were left to dry for two days. They were inoculated with each yeast isolate by streaking aseptically on a plate in duplicates. Incubation was at 28± 2°C for 72h. The growth patterns of the isolates were observed on the medium (Barnett *et al.,* 1990;Kregger van - Rij 1987).

Cellular morphology

Each yeast isolates were stained with lactophenol in cotton blue solution on a clean slide and covered with a clean coverslip. The slides were observed under the microscope using oil immersion objective (x100) as described by Barnett *et al.,* (1990) and Kregger van-Rij (1987).

Pseudomycelium formation

Approximately, 15ml of the potato dextrose agar medium was dispensed into 150ml conical flask containing distilled water and homogenized. This was autoclaved (Okafor, 1978).

Growth at different temperatures

The medium used was YEPD agar medium. A non-inoculated plate with the medium was used as control. Growth along the lines of

streaking was recorded as positive (Onions *et al.*, 1981).

Nitrate assimilation

Approximately, 0.07% (w/v) of KNO_3 solution was used as the source of nitrate in the medium while modified Bacto-yeast carbon base was used as basal medium. Approximately, 15ml of this was dispensed into screw cap tubes and sterilized in the autoclave. Two sets of tubes were prepared for each test organism. The control tube was not inoculated while the other tube was inoculated with the test organism. Filter sterilization of 0.07% (w/v) $KNO_3$was done and 5ml of the solution was aseptically dispensed into each test tube containing the sterile basal medium. Incubated at 30°C for 7 days. Growth was observed in the test tubes by visualization. Incubation was at 30^0C for two weeks and inspection for growth at three days intervals was carried out (Collins *et al.*, 1989).

Urease production

Sufficient 20% urea solution previously sterilized by filtration was then added to give a final concentration of 20%. The bottles were slanted and allowed to solidify. Isolates were then inoculated on the slants. A control of basal medium without urea was inoculated to check that ammonia was produced from urea and not from peptone. The bottles were incubated at 30°C for 7 days. Urease production and subsequent hydrolysis of urea results in the production of ammonia, increase in the pH is shown by colour changes from yellow to pink or red in the conical flask and sterilized at 121°C for 5 minutes, 0.001mg/ml of streptomycin sulphate was added aseptically after sterilization to suppress bacterial growth. Molten PDA was poured into the surface of sterile glass slides, which were suspended by sterile glass rods in sterilized Petri dishes. The agar was allowed to solidify before isolates were streaked onto the glass slides, which were removed with sterile cover slips using sterile forceps. The Petri dishes were incubated for 24h at 30°C. The slides were removed and examined microscopically (using oil immersion objective) for pseudo-mycelium formation which is indicated by filamentous growth of the isolates. A control was set up in the same way but without inoculant (Onions *et al.*, 1981).

Biochemical tests

The isolates were subjected to the following tests for possible identification; Ash content determination, Crude fat determination (Uzogara, 2009), Crude fibre determination, Crude protein, Acid production, Sugar fermentation (Collins *et al.*, 1989).

Ascorbic acid (Vitamin C) visual titration method

Extracting solution

Approximately, 52% of Trichloroacetic acid (TCA), 5g of TCA in 100ml of glan distilled water.

Dye solution

Appr0ximately, 50 mg of 2-, 6-Dichlorophenol - indophenol was weighed and were dissolved in 100ml of hot (85 – 95°C) distilled water, cool and made up to 200ml volume in volumetric flask (A.O.A.C., 2012).

Preparation of sample

Approximately, 5.0g of sample was weighed and sterilized with 80ml of 5% w/v TCA solution, filtered and sample extract were made up to 100ml volume with extracting solution in a volumetric flask.

Assay of extract

An aliquot (20ml) of the 5% w/v TCA extract of the sample was measured into a beaker (150ml beaker) and the sample titrated with the standard dye solution to pink and point.

The ascorbic acid content of the sample was calculated as given below:

$$\text{mg of ascorbic acid Per 100g of sample} = \frac{BCD \times 100}{EF}$$

where B = Sample Titre value
C = Dye factor
D = Volume of sample made up to 100ml
E = Aliquot volume of sample taken to extract
F = Weight of sample used

(A.O.A.C., 2012; Ziadi *et al.*, 2011).

Results and Discussion

The results of microbiological, chemical and biochemical analysis of two types of indigenous palm wine showed that it is acidic and as the days of fermentation increased, the samples with plant extracts tends towards neutral pH.

The total heterotrophic bacterial counts were relatively low in palm wine samples treated with *Vernonia amygdalina*, *Nauclea vandegucluti* and *Euphorbia sp.* compared to those preserved with only one plant preservative and same with palmwine with no preservative at all. Peak heterotrophic bacterial counts were obtained after 24hours fermentation except the sample with no form of preservative (control). This agrees with Okafor (1975b) that observed higher bacterial counts after 24 hours fermentation of palm wine. This suggested a shift in the pH of the medium; that is, palm wine from neutral pH towards acidic pH. A gradual loss of bacterial and fungal viability was noticed as fermentation time increased from 48 hours to 120 hours, that is, increased acidity (Fig. 1 and 2; Table 1–12).

Table.1 Total Heterotrophic counts *Raphia hookeri*

Sample name	Oh	24h	48h	72h	96h	120h
Control	48×10^1	66×10^2	94×10^3	74×10^4	53×10^5	29×10^5
	48×10^1	62×10^2	91×10^3	78×10^4	50×10^5	33×10^5
T0 Bitter leaves	30×10^1	42×10^2	29×10^3	20×10^4	12×10^5	08×10^5
	34×10^1	32×10^2	26×10^3	18×10^4	10×10^3	08×10^3
T1 *Nauclea sp.* (Bark)	36×10^1	48×10^2	40×10^3	30×10^4	20×10^3	16×10^3
	39×10^1	45×10^2	38×10^3	73×10^4	22×10^3	17×10^3
T2 *Nauclea sp.* (leave)	40×10^1	$44x\ 10^2$	35×10^3	27×10^4	19×10^3	11×10^3
	42×10^1	47×10^2	33×10^3	24×10^4	16×10^3	14×10^3
T3 *Euphobia*	28×10^1	34×10^2	29×10^3	20×10^4	15×10^3	09×10^3
	30×10^1	36×10^2	27×10^3	22×10^4	17×10^3	12×10^5
T4 Combined	18×10^1	21×10^2	16×10^3	10×10^4	08×10^3	02×10^3
	16×10^1	22×10^2	19×10^3	10×10^4	06×10^3	03×10^3

Table.2 Coliform counts in *Raphia hookeri*

Sample name	Oh	24h	48h	72h	96h	120h
Control	64×10^1	75×10^2	101×10^3	124×10^4	118×10^5	84×10^5
	$59x\ 10^1$	77×10^2	103×10^3	129×10^4	112×10^5	80×10^5
T0 Bitter leaves	43×10^1	56×10^2	69×10^3	81×10^4	46×10^3	33×10^3
	48×10^1	58×10^2	72×10^3	83×10^4	42×10^3	34×10^5
T1 *Nauclea sp.* (Bark)	43×10^1	49×10^2	53×10^3	59×10^4	39×10^3	27×10^3
	45×10^1	52×10^2	56×10^3	66×10^4	37×10^3	24×10^3
T2 *Nauclea sp.* (leave)	46×10^1	53×10^2	59×10^3	60×10^4	35×10^3	24×10^3
	48×10^1	34×10^2	51×10^3	63×10^4	32×10^3	22×10^3
T3 *Euphorbia*	40×10^1	44×10^2	49×10^3	55×10^4	36×10^3	20×10^3
	38×10^1	47×10^2	53×10^3	58×10^4	38×10^3	22×10^3
T4 Combined	32×10^1	34×10^2	38×10^3	36×10^4	21×10^3	15×10^3
	30×10^1	36×10^2	39×10^3	39×10^4	30×10^3	12×10^3

Table.3 Total Heterotrophic counts in *E. guineensis*

Sample name	Oh	24h	48h	72h	96h	120h
Control	56×10^1	72×10^2	102×10^3	86×10^4	60×10^5	44×10^5
	$60x\ 10^1$	76×10^2	104×10^3	88×10^4	58×10^5	40×10^5
T0 Bitter leave	44×10^1	50×10^2	39×10^3	24×10^4	20×10^3	10×10^3
	44×10^1	52×10^2	38×10^3	27×10^4	22×10^3	12×10^3
T1 *Nauclea sp.* (Bark)	40×10^1	53×10^2	42×10^3	36×10^4	28×10^3	19×10^3
	37×10^1	55×10^2	46×10^3	34×10^4	26×10^3	20×10^3
T2 *Nauclea sp.* (leave)	34×10^1	48×10^2	40×10^3	32×10^4	22×10^3	15×10^3
	32×10^1	46×10^2	38×10^3	28×10^4	20×10^3	17×10^3
T3 *Euphorbia*	30×10^1	39×10^2	33×10^3	24×10^4	19×10^3	09×10^3
	37×10^1	42×10^2	35×10^3	26×10^4	17×10^3	12×10^3
T4 Combined	20×10^1	27×10^2	18×10^3	11×10^4	06×10^3	04×10^3
	22×10^1	29×10^2	19×10^3	12×10^4	08×10^3	03×10^3

Table.4 Coliform counts in *E. guineensis*

Sample name	Oh	24h	48h	72h	96h	120h
Control	69×10^1	84×10^2	118×10^3	138×10^4	109×10^5	93×10^5
	72×10^1	86×10^2	120×10^3	142×10^4	107×10^5	96×10^5
T0 Bitter leave	49×10^1	60×10^2	74×10^3	90×10^4	59×10^3	30×10^3
	52×10^1	63×10^2	76×10^3	88×10^4	58×10^3	32×10^3
T1 *Nauclea sp.* (Bark)	47×10^1	58×10^2	65×10^3	83×10^4	60×10^3	40×10^3
	49×10^1	59×10^2	67×10^3	86×10^4	62×10^3	49×10^3
T2 *Nauclea sp.* (leave)	52×10^1	60×10^2	72×10^3	29×10^4	59×10^3	28×10^3
	53×10^1	63×10^2	74×10^3	88×10^4	57×10^3	26×10^3
T3 *Euphorbia*	36×10^1	49×10^2	63×10^3	74×10^4	49×10^3	24×10^3
	34×10^1	52×10^2	65×10^3	76×10^4	52×10^3	22×10^3
T4 Combined	29×10^1	38×10^2	41×10^3	44×10^4	24×10^3	19×10^3
	27×10^1	40×10^2	42×10^3	46×10^4	26×10^3	18×10^3

Table.5 Yeast counts in *Raphia hookeri*

Sample name	Oh	24h	48h	72h	96h	120h
Control	69×10^1	93×10^2	133×10^3	156×10^4	109×10^5	84×10^5
	68×10^1	92×10^2	136×10^3	160×10^4	109×10^5	83×10^5
T0 Bitter leave	60×10^1	79×10^2	58×10^3	39×10^4	30×10^3	24×10^3
	58×10^1	88×10^2	54×10^3	42×10^4	27×10^3	20×10^3
T1 *Nauclea sp.* (Bark)	60×10^1	80×10^2	61×10^3	43×10^4	29×10^3	21×10^3
	58×10^1	83×10^2	63×10^3	45×10^4	26×10^3	19×10^3
T2 *Nauclea sp.* (leave)	55×10^1	63×10^2	50×10^3	39×10^4	25×10^3	19×10^3
	52×10^1	65×10^2	54×10^3	36×10^4	27×10^3	18×10^3
T3 *Euphorbia*	44×10^1	49×10^2	40×10^3	30×10^4	24×10^3	15×10^3
	48×10^1	43×10^2	42×10^3	32×10^4	20×10^3	16×10^3
T4 Combined	30×10^1	39×10^2	27×10^3	23×10^4	16×10^3	10×10^3
	31×10^1	42×10^2	29×10^3	20×10^4	18×10^3	08×10^3

Table.6 Yeast counts in *E. guineensis*

Sample name	Oh	24h	48h	72h	96h	120h
Control	55×10^1	82×10^2	104×10^3	123×10^4	94×10^5	63×10^5
	$60x\ 10^1$	80×10^2	107×10^3	126×10^4	97×10^5	68×10^5
T0 Bitter leaves	49×10^1	63×10^2	39×10^3	28×10^4	19×10^3	10×10^3
	48×10^1	60×10^2	41×10^3	27×10^4	$20x\ 10^3$	12×10^3
T1 *Nauclea sp.* (Bark)	44×10^1	59×10^2	33×10^3	24×10^4	18×10^3	09×10^3
	42×10^1	56×10^2	35×10^3	25×10^4	16×10^3	08×10^3
T2 *Nauclea sp.* (leave)	46×10^1	57×10^2	30×10^3	20×10^4	14×10^3	08×10^3
	49×10^1	56×10^2	28×10^3	23×10^4	13×10^3	06×10^3
T3 *Euphorbia*	40×10^1	59×10^2	43×10^3	37×10^4	28×10^3	19×10^3
	39×10^1	60×10^2	40×10^3	35×10^4	25×10^3	20×10^3
T4 Combined	29×10^1	40×10^2	26×10^3	18×10^4	12×10^3	08×10^3
	32×10^1	42×10^2	24×10^3	19×10^4	13×10^3	06×10^3

Table.7 Nutritional composition of preserved palm wine *Raphia hookeri*

	Crude protein %	Moisture %	Fat (lipid) %	Crude fiber %	Ash %	Vitamin E %
Control	0.052	98.44	0.05	0.24	0.06	7.86
T0	0.30	96.94	0.07	0.42	0.12	9.98
T1	0.22	96.94	0.06	0.63	0.18	8.75
T2	0.26	96.82	0.09	0.82	0.16	9.66
T3	0.35	96.38	0.12	0.12	0.19	9.89
T4	0.40	95.64	0.18	0.18	0.23	12.43

Table.8 Nutritional composition of preserved palm wine *E. guineensis*

	Crude protein %	Moisture %	Fat (lipid) %	Crude fiber %	Ash %	Vitamin C %	Carbohydrate %
Control	0.048	98.68	0.04	0.028	0.05	7.36	1.19
To	0.26	97.36	0.05	0.038	0.10	3.94	2.19
T_1	0.20	97.08	0.07	0.52	0.16	9.24	2.44
T_2	0.22	96.74	0.05	0.074	0.14	9.39	2.78
T_3	0.32	96.54	0.10	0.96	0.17	10.22	2.74
T_4	0.44	95.36	0.16	0.15	0.21	13.24	3.68

Table.9 Micro-morphological and biochemical characterization of heterotrophic bacterial population

Isolate code	Colour / pigment	Gram reaction	Cellular morphology	Catalase test	Oxidase test	Indole test	Motility test	methyl Red	Voges Proskauer	Urease activity	Citrate utilization	Gelatin hydrolysis	Starch hydrolysis	Coagulase test	Spore test	NO_3 reduction	Glucose	Sucrose	Lactose	Xylose	Sorbitol	Salicin	Mannitol	Maltose	Aribinose	Raffinose		Probable Identity
CON1	Cream	+	Rods	+	+	-	+	-	-	-	-	+	-	-	+	-	+	+	+	-	-	-	+	-	+	-		*Bacillus laterosporus*
CON2	Cream	-	Rods	+	+	+	+	-	-	+	-	-	-	-	-	+	+	-	+	-	-	-	-	-	-	-		*Akaligenes eutrophis*
CON3	Pinkish	-	Rods	+	-	-	+	+	-	+	+	-	-	-	-	-	+	+	+	+	+	-	+	+	+	-		*Citrobacter freundii*
CON4	Orange	+	Coci	+	-	-	-	-	+	+	-	-	-	+	-	+	+	+	+	-	-	+	+	+	-	-		*Staphylococcus aureus*
CON5	Cream	+	Rods	+	+	-	+	-	+	-	+	+	+	-	+	-	+	+	+	-	-	-	-	+	-	-		*Bacillus cereus*
CON6	Black	-	Rods	+	-	+	+	+	-	-	-	-	-	-	-	+	+	-	+	+	+	+	+	+	+	+		*Escherichia coli*
T01	Pinkish	-	Rods	+	-	-	+	+	-	-	+	-	-	-	-	+	+	-	-	+	-	-	+	+	-	-		*Salmonella bongori*
T02	Yellow	+	Coci	+	+	-	-	-	-	+	-	+	-	-	-	-	+	+	-	+	-	-	-	-	-	-		*Micrococcus luteus*
T03	Cream	-	Rods	+	-	+	+	-	+	-	-	+	-	-	-	+	+	-	-	+	-	+	+	-	+	-		*Erwinia clorysanthemi*
T1 1	Cream	+	Rods	+	+	-	+	-	+	-	+	+	+	-	+	+	+	+	+	-	-	-	+	-	+	-		*Bacillus subtilis*
T1 2	Cream	+	Rods	+	-	-	+	-	+	-	+	+	+	-	+	+	+	+	+	-	-	-	+	-	+	-		*Bacillus licheniformis*
T1 3	Yellow	+	Coci	+	-	-	-	-	+	+	-	+	-	-	-	+	+	+	+	-	-	-	-	+	+	-		*Staphylococcus epidermidis*
T2 1	Cream	+	Rods	+	+	-	+	-	+	-	+	+	+	-	+	+	+	+	+	+	-	+	+	+	+	+		*Bacillus polymyxa*
T2 2	Yellow	-	Rods	+	-	-	+	+	-	-	+	+	+	-	-	-	+	+	+	+	+	+	+	+	-	-		*Xanthomonas campestris*
T2 3	Pink	-	Rods	+	-	-	+	-	+	-	+	-	-	-	-	+	+	-	-	+	-	-	+	+	-	-		*Enterobacter amnigenus*
T3 1	Black	-	Rods	+	-	-	+	-	-	-	+	+	-	-	-	-	+	-	-	-	-	-	-	+	-	-		*Salmonella arizonae*
T3 2	Cream	+	Rods	+	+	-	+	-	+	-	+	+	+	-	+	+	+	+	+	-	-	-	+	-	+	-		*Bacillus subtilis*
T3 3	Cream	-	Rods	+	-	-	-	-	-	+	+	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-		*Acinetobacter iwoffi*
T4 1	Cream	+	Rods	+	+	-	+	+	-	-	-	+	-	-	+	+	+	-	-	+	-	-	+	-	-	-		*Bacillus brevis*

Table.10 Micro-morphological and biochemical characterization of bacterial isolates

Isolate code	Colour / pigment	Gram reaction	Cellular morphology	Catalase test	Oxidase test	Indole test	Motility test	Methyl Red	Voges Proskauer	Urease activity	Citrate utilization	Gelatin hydrolysis	Starch hydrolysis	Casein hydrolysis	No_3 reduction	Glucose	Sucrose	Lactose	Xylose	Sorbitol	Inorsitol	Mannitol	Maltose	Raffinose	Probable Identity
CON1	Pink	-	Rods	+	-	-	+	-	+	-	-	+	-	-	+	+	+	+	+	+	+	+	+	+	*Klebsiella aerogenes*
CON2	Pink	-	Rods	+	-	-	-	-	-	-	+	-	-	-	-	+	-	+	-	-	-	+	-	-	*Acinetobacter mallee*
CON3	Pink	-	Rods	+	--	-	+	-	+	+	+	-	+	-	-	+	+	-	+	+	+	+	+	+	*Enterobacter cloacae*
CON4	Pink	+	Cocii	+	-	-	+	-	-	-	+	-	-	-	+	+	+	-	-	-	-	-	-	-	*Alkaligenes faecalis*
CON5	Pink	-	Rods	+	-	-	-	-	+	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+	*Klebsiella preumoniae*
T01	Black	-	Rods	+	-	+	+	+	-	-	-	-	-	-	+	+	+	-	+	+	-	+	+	+	*Escherichia coli*
T02	Pink	+	Cocii	+	-	+	-	-	+	+	+	+	+	-	+	+	+	+	+	-	-	+	+	+	*Klebsiella oxytoca*
T03	Pink	-	Rods	+	-	-	+	-	+	-	-	-	-	-	+	+	+	+	+	-	-	+	+	-	*Enterobacter amnigenus*
T1 1	Pink	-	Rods	+	-	+	+	-	+	-	-	+	-	-	-	+	-	-	+	-	-	+	-	-	*Enwinia chrysanthenic*
T1 2	Pink	-	Rods	+	-	-	+	+	-	+	+	-	-	-	-	+	+	+	+	+	-	+	+	-	*Citribacter freundii*
T1 3	Pink	+	Cocii	+	+	+	+	-	-	+	-	-	-	-	+	+	-	+	-	-	-	-	-	-	*Alcaligenes eutrophs*
T2 1	Black	-	Rods	+	-	-	+	-	-	-	+	+	-	-	-	+	-	-	-	-	-	-	+	-	*Salmonella arizonae*
T2 2	Black	-	Rods	+	-	-	+	+	-	-	+	-	-	-	+	-	-	-	+	-	-	+	+	-	*Salmonella bongori*
T2 3	Brown	-	Rods	+	+	-	+	-	-	-	+	+	-	-	+	+	+	-	-	-	-	+	+	+	*Pseudomonas aeruginosa*
T3 1	Pink	-	Rods	+	-	-	+	+	+	-	+	-	-	-	+	+	+	-	+	+	+	+	+	+	*Enterobacter agglomerans*
T3 2	Brown	-	Rods	+	+	-	+	-	-	-	+	+	-	-	+	+	+	-	-	+	-	+	-	-	*Pseudomonas chlororaphis*
T3 3	Pink	-	Rods	+	-	-	+	+	+	-	+	-	-	-	+	+	+	-	+	+	+	+	+	+	

KEY: + = Positive; - = Negative

Staphylococcus

Table.11 Micro-morphological and biochemical characterization of bacterial isolates

Isolate code	Colour / pigment	Gram reaction	Cellular morphology	Catalase test	Oxides test	Indole test	Motility test	MR-methy/Red	VP-Voges Proskauer	Urease activity	Citrate utilization	Starch hydrolysis	Gelatin hydrolysis	Casein hydrolysis	Coagulase test	No_3 reduction	Growth in 10% NaCl	Glucose	Sucrose	Xylose	Salicin	Maltose	Mannitol	Raffinose	Galatine	Arabinose		Probable Identity
C1	Orange	+ve	Cocii	+	-	-	-	-	+	+	-	-	-	-	+	+	+	+	+	-	-	+	+	+	+	-	+	*Staphylococcus aureus*
C2	White	+ve	Cocii	+	+	-	-	-	+	-	-	-	-	-	-	-	+	+	+	-	+	+	-	+	-	+	+	*Staphylococcus albrus*
C3	Yellow	+ve	Cocii	+	-	-	-	-	+	+	-	-	+	-	-	-	+	+	+	-	-	+	-	-	-	+	+	*Staphylococcus epidermidis*
C4	Orange	+ve	Cocii	+	-	-	-	-	+	+	-	-	-	-	-	+	+	+	+	+	+	-	+	-	-	-	+	*Staphylococcus simulant*
T05	Yellow	+ve	Cocii	+	-	-	-	-	+	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	-	+	+	*Staphylococcus ariethae*
T06	Yellow	+ve	Cocii	+	-	-	-	-	-	-	+	-	+	-	-	-	-	+	+	-	-	+	-	-	-	-	-	*Micrococcus varians*
T07	Red	+ve	Cocii	+	-	-	+	-	+	-	-	-	-	-	-	+	-	+	-	+	-	+	+	-	-	-	-	*Micrococcus roseus*
T08	Yellow	+ve	Cocii	+	+	-	-	-	+	-	+	-	-	-	-	-	-	+	+	-	-	+	-	-	-	-	-	*Micrococcus kristinae*
T1 9	Yellowish	+ve	Cocii	+	+	-	-	-	+	+	-	-	+	-	-	-	-	+	+	-	-	+	-	-	-	-	-	*Micrococcus candidus*
T1 10	Yellow	+ve	Cocii	+	+	-	-	-	-	+	-	-	+	-	-	-	-	+	+	-	-	-	-	-	-	-	-	*Micrococcus luteus*
T1 11	Cream	+ve	Cocii	+	-	-	-	-	+	+	-	-	-	-	-	+	+	+	-	-	-	+	+	-	-	-	+	*Staphylococcus carnosus*
T2 12	Cream	+ve	Cocii	+	-	-	-	-	+	+	-	-	-	-	-	+	+	+	+	-	-	+	-	-	-	-	-	*Staphylococcus hominis*
T2 13	Orange	+ve	Cocii	+	-	-	-	-	+	+	-	-	-	-	+	+	+	+	+	-	-	+	+	+	+	-	+	*Staphylococcus aureus*
T2 14	White	+ve	Cocii	+	+	-	-	-	+	-	-	-	-	-	-	-	+	+	+	-	+	+	-	+	-	+	+	*Staphylococcus alibus*
T3 15	Orange	+ve	Cocii	+	-	-	-	-	+	+	-	-	-	-	+	+	+	+	+	-	-	+	+	+	+	-	+	*Staphylococcus aureus*
T3 16	Orange	+ve	Cocii	+	-	-	-	-	+	+	-	-	-	-	-	+	+	+	+	+	+	-	+	-	-	-	+	*Staphylococcus simulant*
T3 17	Red	+ve	Cocii	+	-	-	+	-	+	-	-	-	-	-	-	+	-	+	-	+	-	+	+	-	-	-	-	*Micrococcus roseus*
T4 18	Orange	+ve	Cocii	+	-	-	-	-	+	+	-	-	-	-	+	+	+	+	+	-	-	+	+	+	+	-	+	*Staphylococcus aureus*
T4 19	Yellow	+ve	Cocii	+	+	-	-	-	-	+	-	-	+	-	-	-	-	+	+	-	-	-	-	-	-	-	-	*Micrococcus luteus*

KEY: + = Positive; - = Negative

Table.12 Micro-morphological and biochemical characterization of yeasts isolates

Isolate code	Colour / pigment	Cellular morphology	Motility test	Catalase test`	No_3 reduction	Urease activity	Ascospore formation formation	Pseudomycelium	Glucose	Xylose	Raffinore	Sucrose	Fructose	Lactose	Maltose	Mannitol	Melibose	Trehalose		Arabinose	Probable Identity
CON1	Cream	Oval, budding	+	+	-	-	+	-	+	+	+	+	+	-	+	-	-	+	+	+	*Saccharomyces cerevisiae*
CON2	Cream	Oval	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	*Kluyveromyces maxiams*
CON3	Cream	Oval	+	+	+	-	-	-	+	-	-	+	-	-	+	-	-	-	-	-	*Hansenula anomala*
CON4	Red	Elongated	+	+	-	+	-	-	+	-	-	+	-	-	+	-	-	-	-	-	*Rhodotorula graminis*
T01	Cream	Oval, budding	+	+	-	-	+	-	+	+	+	+	+	-	+	-	-	+	-	+	*Saccharomyces cerevisiae*
T02	Cream	Oval, small	+	+	-	-	-	-	+	-	-	+	+	-	+	-	-	-	-	-	*Totulopsis delbrueckii*
T03	Cream	Oval, budding	+	+	-	-	+	-	+	-	-	-	+	-	+	-	-	-	-	-	*Saccharomyces rouxii*
T04	Pink/Red	Elongated	+	+	+	+	-	-	+	-	+	+	-	-	+	-	-	+	+	-	*Rhodotorula glutinis*
T1 1	Cream	Oval, small	+	+	-	-	-	+	+	+	-	-	-	-	-	-	-	-	+	-	*Candida rugosa*
T1 2	Cream	Oval, budding	+	+	-	-	+	-	+	+	+	+	+	-	+	-	-	+	+	+	*Saccharomyces cerevisiae*
T1 3	Cream	Oval, budding	+	+	-	-	+	-	+	+	+	+	+	-	+	-	-	+	+	+	*Saccharomyces cerevisiae*
T1 4	Cream	Cylindrical	+	+	-	-	-	+	+	+	-	+	-	-	+	-	-	+	+	-	*Candida albicans*
T2 1	Pink	Elongated	+	+	+	+	-	-	+	-	+	+	-	-	+	-	-	-	-	-	*Rhodotorula mucilaginous*
T2 2	Cream	Oval, small, budding	+	+	-	-	+	-	+	-	-	+	-	-	+	-	-	+	-	-	*Pichia amgusta*
T2 3	Cream	Ellipsoidal	+	+	-	-	-	-	+	-	-	+	+	-	-	-	-	-	+	-	*Torulopsis stellata*
T2 4	Cream	Round, budding	+	+	-	-	+	-	+	-	-	+	-	-	-	-	-	+	-	-	*Pichia ohmeri*

KEY: + = Positive; - = Negative

Fig.1 Determination of pH values *Raphia hookeri*

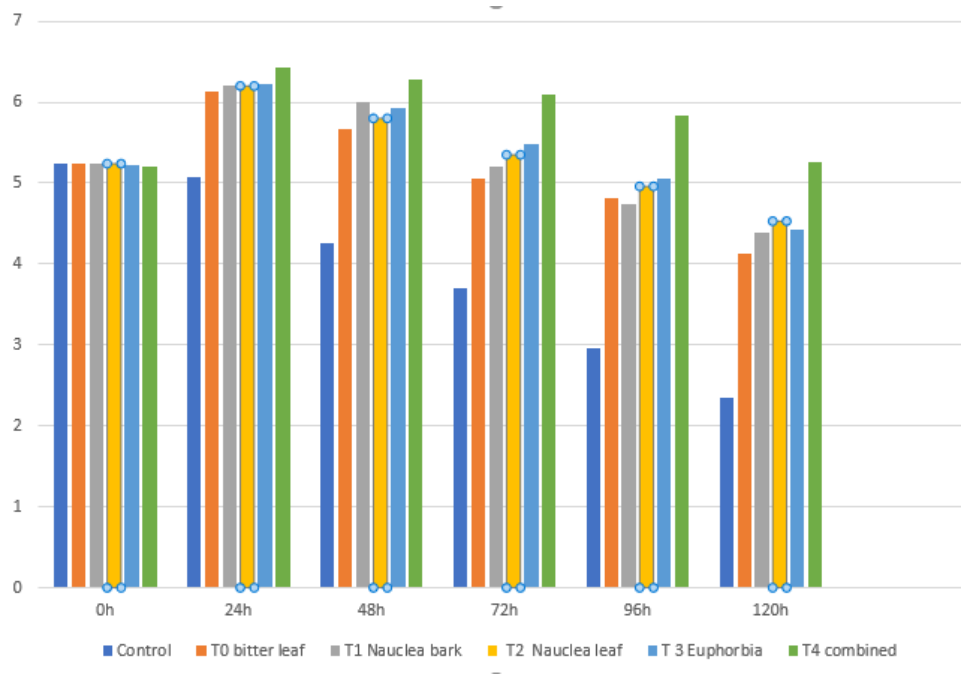
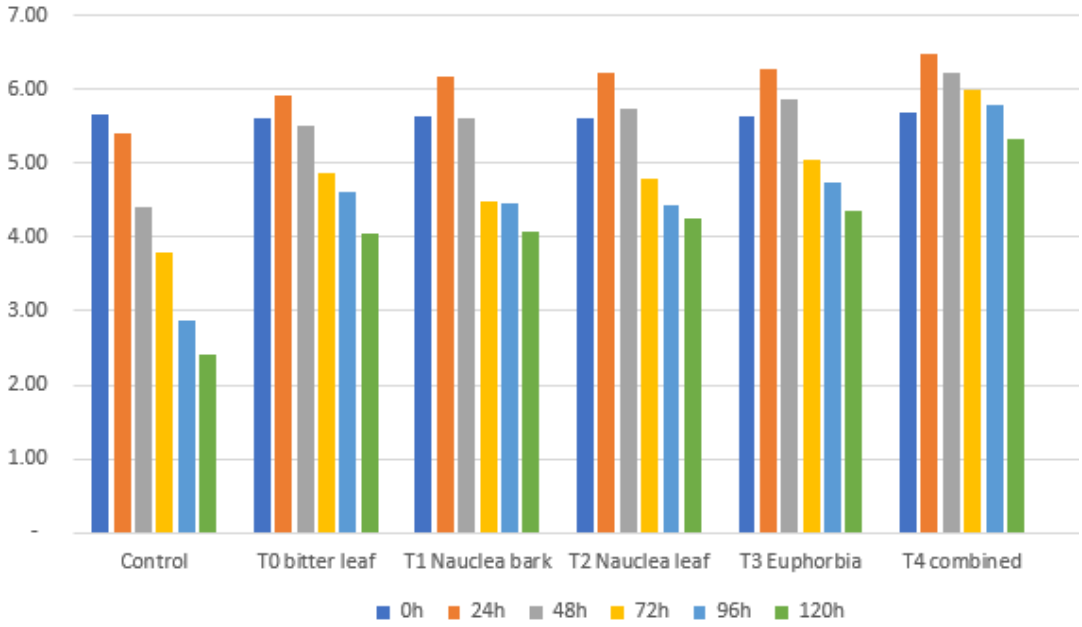


Fig.2 Determination of pH values *E. guineensis*



Seven probable isolates were identified. Bacterial genera which included *Staphylococcus*, *Micrococcus*, *Bacillus*, *Lactobacillus*, *Brevibacterium*, *Escherichia*, *Klebsiella*, *Acinetobacter*, *Enterobacter*, *Alcaligenes* and yeast isolates identified to

belong to the genera *Saccharomyces.* The presence of non-Saccharomyces yeasts among the fermenting organisms corroborated the findings of Moreira *et al.,* (2011). The non-Saccharomyces have been found to incorporate esters, fatty acids and other organic compounds to wine which is not unexpected (Table, 12) since palm wine fermentation is often spontaneous (Moreira *et al.,* 2011). The non-Saccharomyces yeasts are known to have relatively high ethanol-tolerance and osmo-tolerance (Nwachukwu *et al.,* 2006). The occurrence of these microbial isolates in the palm wine samples supported the reports made by Faparusi and Bassir (1972), Okafor (1975a, b) and Ikenebomeh and Omayuli (1988), and lends more weight to the present findings. The isolation of *Micrococcus* sp. from fermenting palm wine poses health implications which might have been due to the exposure of freshly tapped palm sap, which supported the report of Ikenebomeh and Omayuli (1988), that showed various forms of pathogenic bacteria associated with exposed palm wine. The frequent gastro-intestinal problems associated with drinking palm wine after 24 hours could be attributed to the presence of pathogenic bacteria in palm wine. The gradual reduction in viability of microbial isolates in palm wine preserved with plant material could also be attributed to the presence of bio-active components present in the *V. amygdalina*, *N. vandegucluti* and *Euphobia* which corroborated the work of Ogbulie*et al.,* (2007) which showed antibacterial effects of medicinal plants against pathogens(Akujiobi *et al.,* 2004). The addition of plant preservatives (Table 9, 10) produced consequences that corroborated previous findings of improved organoleptic properties, crude protein and total carbohydrate content in the different types of palm wine (Njoku *et al.,* 1991).

In conclusion, this study therefore showed that plants preservatives have potentials of extending the shelf- life of these two types of indigenous palm wine and by extension that of any other alcoholic beverage. The use of these plant extracts did not affect the nutritional values and organoleptic properties as well as acceptability to the traditional African communities, thus, contributing to the search for low-cost preservative methods for palm wine storage. It raises strong hope for the industrial ethanol fermentation production at reduced costs.

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