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Stabilization of Local Drink "Tchakpalo" produced in Benin by addition of Essential Oil Extracted from Fresh leaves of *Cymbopogon citratus*

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Abstract

The study is a part of the valuation of the local beer tchakpalo. It was to evaluate the preservative effect of essential oil extracted from fresh leaves of Cymbopogon citratus for the stabilization of this drink. Essential oil was extracted by hydrodistillation with a Clevenger-type apparatus and analyzed by gas chromatography and gas chromatography coupled with mass spectrometry. The efficacy of this oil was measured against microrganisms that cause fermentation and adulteration of the drink (Saccharomyces cerevisiae, Aspergillus niger, Fusarium oxysporum, Penicillium camembertii) and pathogenic microorganisms which could contaminate the product, Escherichia coli ATCC 25922 and Staphylococcus aureus ATCC25923 by microdilution and agar diffusion methods. The yield of extraction of leaves essential oil of this plant was 1.70 ±0.02%. The major components of the essential oil of Cymbopogon citratus were geranial (41.3%), neral (33.0%) and myrcene (10.4%). The minimal inhibitory concentrations determined and the minimual bactericidal concentrations calculated for this oil allowed to value its antibiotical power. The essential oil of Cymbopogon citratus possessed antibiotical potency against Escherichia coli ATCC 25922 and Staphylococcus aureus ATCC 25923. It had fungicidal activity against Saccharomyces cerevisiae at 0.48 mg/mL, P. camembertii at 500 ppm, Aspergillus niger and F. oxysporum at 1100 ppm. The addition of this essential oil to Tchakpalo helped make this drink stable for a period of ten weeks. However, it should be noted that the conservative action of this oil is less pronounced than that of citric acid tested for the same purpose. Essential oil of Cymbopogon citratus could be a potential substitute for synthetic chemical additives used in the preservation of beverages in general. Its use as biopreservative of the food deserves to be promoted.

Keywords: volatile extract, pathogens, beverage, preservation, antimicrobial activity.

Introduction

Hunger and malnutrition remain the major problems of our societies, in particular, populations in developing countries where the concept of food security remains a luxury[1]. To contribute to the fight against food insecurity, we should increase agricultural production and value to local products through the judicious use of technical knowledge. The man uses for millenium the fermentation to obtain improved nutritional value foods[2-3]. In Africa, some cereals such as sorghum, maize and millet are often transformed into beverage whose manufacture includes an essential step of alcoholic fermentation [4]. These drinks have a central role in peoples' cultures. In Benin and like other countries of the subregion, sorghum is usually transformed into a traditional beer called "tchakpalo". Initially produced in the center of the country, this drink has spread throughout the country and especially in the economic capital where it knows a boom [5]. In its usual production technology, we note the presence of a double fermentation: an alcoholic one which combines natural lactic fermentation. Over time, the conditions of the production have not changed. The drying of germinated sorghum grain is made in full air, in border of the ways. Moreover, the production process suffers from a crucial lack of measuring instruments and precision, good hygiene practices and wort was inoculated with the yeast from the previous fermentation whose the hygienic quality was often not guaranteed[5]. The beverage thus obtained was unstable. It is then important to improve production and conservation technology of this product in order to preserve its nutritional and merchantable qualities and to reduce the risk of food intoxination. Chemical preservatives such as benzoates, sulphites, α-tocopherol, calcium chloride and citric acid were usually used for food preservation in general and especially drinks[6]. However, at short or long-term, these synthetic chemical products could be very toxic, with risks of mutagenicity, chromosomal aberrations and cancer[6-7]. Due to the resurgence of the harmful effects of these chemical substances on the human health, the use of essential oils generally recognized as safe (GRAS) as bioconservatives agents of tchakpalo could be a credible alternative[8]. Indeed, essential

oils possess antimicrobial activities and are without major effects on the environment and human health[8-11]. According to Ohno *et al*.[12], the essential oil of *Cymbopogon citratus* had antimicrobial activity against *Helicobacter pylori*. Although the antimicrobial properties of this oil has been studied, its application as a natural preservative in food were very few developped. The efficacy of this oil as biopreservative of Tchakpalo must be verified in order to the valorization of this product. The objective of this study was to measure antimicrobial activities of essential oil of *Cymbopogon citratus* against microorganisms that cause fermentation and spoilage of Tchakpalo and pathogenic microorganisms which could contaminate this drink and to access potential biopreservative of this oil compared to citric acid for the stabilization of tchapalo.

Material and Methods

Collection of plant material: Samples of red variety of sorghum (*Sorghum bicolor* (L.) Moench) were purchased from local markets in Glazoué (center of Benin) and used for the preparation of the traditional beer Tchakpalo. Fresh leaves of *Cymbopogon citratus* identified by Dr Yedomonhan of National Herbarium of Benin were collected at Ouémé (south Benin) on ferralitic soil and its essential oil was extracted by hydrodistillation using a clevenger apparatus type. Oil recovered was dried over anhydrous sodium sulfate and stored at +4°C until it was used.

Identification of chemical components of *Cymbopogon citratus* essential oil: Quantitative and qualitative analyses of the essential oil of *Cymbopogon citratus* were carried out by gas chromatography/flame ionization detection (GC/FID) and gas chromatography/mass spectrometry (GC/MS).

GC/FID analyses were performed using a varian CP-3380 GC equipped with a DB1 (100% dimethylpolysiloxane) fitted with a fused silica capillary column (30 m x 0.25 mm, film thickness 0.25 µm) and supelcowax 10 (polyethylene glycol) fused capillary column (30 m x 0.25 mm, film thickness 0.25 µm); temperature program 50°-200°C at 5°C/min, injector and detector respectively at 220°C and 250°C, carrier gas N_2 at a flow rate of 0.5 mL.min^{-1}. Diluted samples (10/100, v/v, in methylene chloride) of 2.0 µL were injected manually in a split mode (1/100). The percentage compositions were obtained from electronic integration measurements without taking into account relative response factors. The linear retention indices of the components were determined relatively to the retention times of a series of *n*-alkanes (C_9-C_{20}).

GC/MS analyses were performed using a Hewlett Packard apparatus equipped with a HP1 fused silica column (30 m x 0.25 mm, film thickness 0.25 µm) and interfaced with a quadruple detector (Model 5970). Column temperature was programmed from 70° to 200°C at 10°C/min; injector temperature was 220°C. Helium was used as carrier gas at a flow rate of 0.6 mL.min^{-1}, the mass spectrometer was operated at 70 eV. 2.0 µL of diluted samples (10/100, v/v, in methylene chloride) were injected manually in the split mode (1/100).

The identification of individual compounds was based on the comparison of their relative retention times with those of authentic samples on the DB5 column and by matching the linear retention indices and mass spectra of peaks with those obtained from authentic samples and/or the NBS75K.L and NIST98.L libraries and published data[13-15]**.**

Strains tested: they were constituted of predominant yeast (*Saccharomyces cerevisiae*) of tchakpalo identified by Gram staining according to keys of Guiraud and Galzy[16] and moulds (*Aspergillus niger*, *Fusarium oxysporum*, *Penicillium camembertii)* which contamined this drink isolated from DBRC medium by dilution method and purified by streaking onto malt extract agar (MEA) and czapeck yeast autolysate (CYA) agar before identification based both on macroscopic characters (colony growth, colony diameter) and microscopic characters using the identification schema of Samson *et al*. [17]. Pathogenic bacteria such as *Escherichia coli* ATCC25922 and *Staphylococcus aureus* ATCC 25923 obtained from National Laboratory of the Ministry of Public Health of Benin which could contamine this drink were also tested.

Biological assays: *In vitro* Antifungal tests against moulds: The moulds detected after examination and identification then, are transplanted (subcultured) using a disc of 6 mm in diameter which carries spores from the anamorph mould on the surface of Petri dish containing the former medium Yeast Extract Glucose Agar containing tested essential oils at different concentrations or no (positive control). The moulds subcultured were incubated at 25 ± 1°C. The mycelial growth was appreciated every day by measuring the average of two perpendicular diameters passing by the middle of the disc, from the first day till the seventh one at least 7 days[18]. The antifungal activity of this oil was evaluated by the following equation: I= [1-(d/dc)] × 100. I: index antifungal; d: diameter of growth of Petri dish treated out of essential oil; dc: diameter of growth

Nature of essential oil activity against moulds: With the experimental concentrations where no growth or germination was observed, the fungistatic or fungicidal activity was tested. This test consisted in taking the mycelial disc not germinated at the end of the incubation of the Petri dish and reintroducing it in a new culture medium (former one) without natural extract. If the mycelial growth is always inhibited, the fungicidal activity of the natural extracts and in the contrary case, it's spoken about fungistatic activity of the essential oil[19].

In Vitro* Antifungal tests against predominant yeast *Saccharomyces cerevisiae: For *Sacchromyces cerevisiae* specie cultured as bacteria, the method performed is the one described by Bajpai *et al*. [20-21] and reported by Yèhouenou *et al*.[19].

In vitro* Antibacterial assay: *Minimum inhibitory concentration (MIC)-broth microdilution method: To

determine the MIC, broth microdilution method proposed by Bajpai *et al.* [20] and reported by Yèhouenou *et al.*[19] were used. The microdilutions on 96 well plates were used with MHB and 0.02 g/L phenol red. Essential oil and MHB constitute the negative control. The positive one is bacteria strain with MHB. The microplates were incubated at 37 ± 1°C for 24 h, covered with a parafilm paper.

Minimal Bactericidal Concentration (MBC): MBC were appreciated by method performed by Oussou *et al.* [22] reported respectively by Kpadonou *et al.* [23]. To determine the MBC, each microliter-plate well content 50 µl in which no color change occurred, the mixture of Eo and the strain was isolated on sterile MHA poured in Petri dishes. These plates were incubated at 37°C for 24 h. The MBC is the lowest concentration of essential oil which 99.9% of the microorganisms were killed. The tests were carried out in triplicate.

Test in Tchakpalo food system for biopreservation capacity of *Cymbopogon citratus* essential oil: For the improvement of the stability of Tchakpalo, essential oil of c*ymbopogon citratus* was added to this drink after its production with empiric manufacturing process and stored as described by figure-1. Citric acid was also used as reference conservative. Physico-chemical, microbiological analyses were conducted on local beer Tchakpalo during its storage and sensory analysis was done on this product at the end of duration of the experimentation.

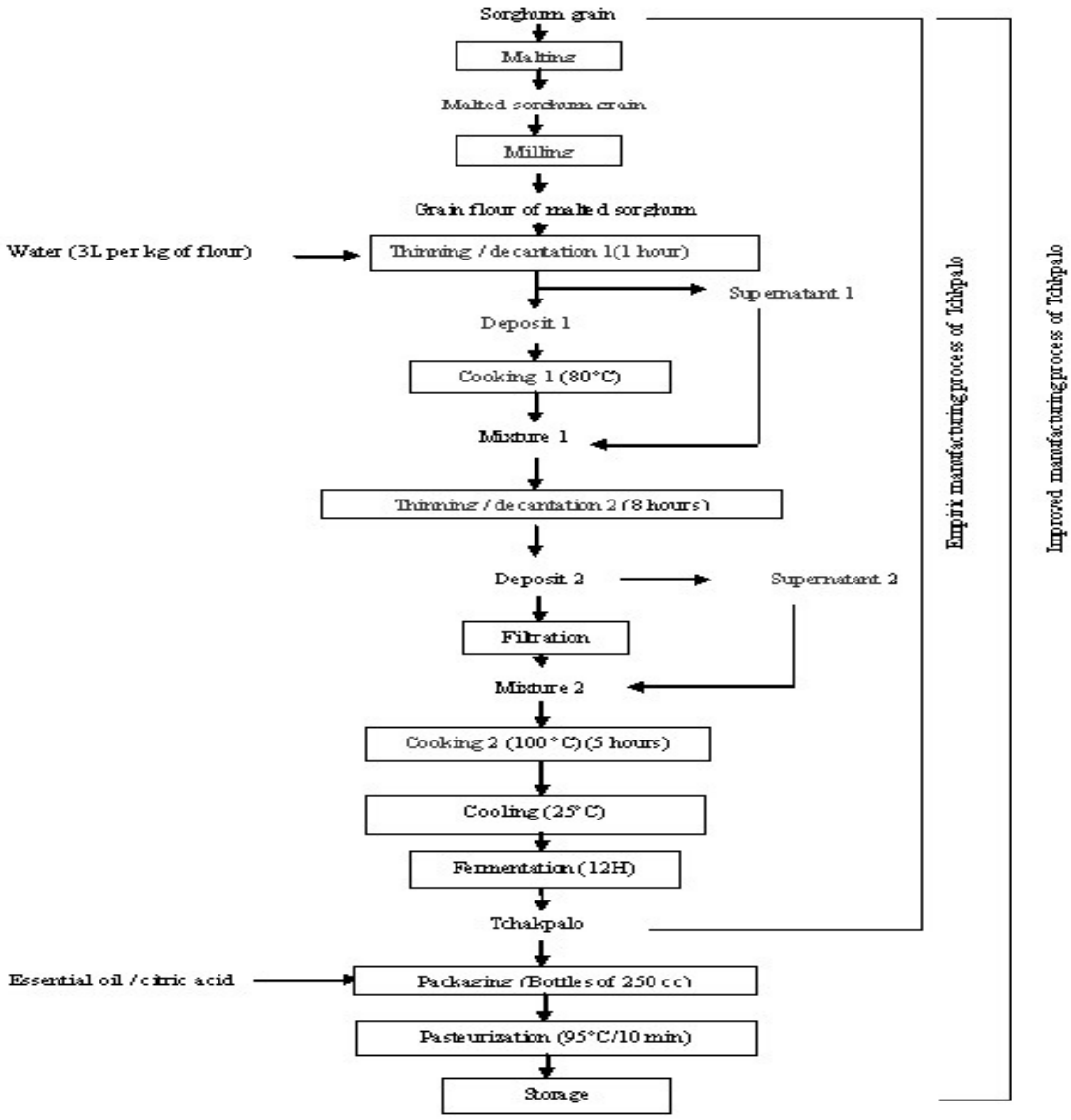

Figure-1
Manufacturing processes of Tchakpalo

The different samples of tchakpalo obtained are divided into three lots described as follows: Lot E0: negative control; it's pasteurized and bottled tchakpalo without the addition of essential oil; Lot E1: positive control ; samples that received a dose of 0.1% of citric acid; Lot E2: the samples that received a dose of 0.1% of essential oil of *Cymbopogon citratus.*

Physico–chemical analysis: The pH was measured with a digital pH-meter (HANNA HI 98129). Acidity of samples, expressed as citric acid content per unit of volume, was determined by titration with 0.01 mol/L of sodium hydroxide solution, using phenolphthalein as indicator [24]. The Brix was measured by a refractometer (ATAGO, Japan). Dry matter and moisture content were determined according to AOAC[25] method, the total sugar of the drink were determined by the method of Dubois *et al*.[26] and alcohol is measured using an alcoholmeter in a test tube, taking into account the temperature of the wort.

Microbiological analysis: Some parameters such as total aerobic mesophilic germs, yeasts and moulds were determined according respectively to standard NF V 08-05 with plate count agar medium PCA OXOID CM0463 and standard ISO 7954:1987 (F) with sabouraud dextrose agar medium OXOID CM0041.

Sensory analysis: The sensory characters of the different beers were evaluated by 30 untrained judges but familiar with *Tchakpalo*. The drinks were presented to the panellists in a random order using 3–degit random number codes and were asked to indicate their preference in a hedonic preference test[27-28]. Attributes (flavor and taste) were assessed.

Statistical analysis: Data from three independent replicate trials were subjected to statistical analysis using Statistica version 6.0. Differences between means were tested using Z-test.

Results and Discussion

Chemical composition of essential oil of ***Cymbopogon citrates:*** The chemical composition of *Cymbopogon citratus* essential oil with yied equal to 1.70 ± 0.02% is presented in table -1. Twenty six components which represented 98.1 % of the total oil were identified in the essential oil. The main components were geranial (41.3%), neral (33.0%) and myrcene (10.4%). The other minor compounds in significant percent were geraniol (6.6) and geranyl acetate (2.4%).

In Vitro antimicrobial properties of essential oil of *Cymbopogon citrates:* Antifungal activity: The figures-2-4 present the reduction rate, by the essential oil of *Cymbopogon citratus*, of the mycelial growth of of *Aspergillus niger*, *Fusarium oxysporum* and *Penicillium camembertii* depending on the incubation time. We note that on *Aspergillus niger*, for the concentration of 500 ppm, the rate of reduction increased progressively from 61.290% in the first day to 85% on the third day before decreasing from this value until 28.571% on the tenth day. At concentrations of 1100 ppm and 2500 ppm of the essential oil, the reduction rate increased from 61.290% after 24 hours of incubation until 92.857% in the tenth day. On *Fusarium oxysporum* at the concentration of 500 ppm of *Cymbopogon citratus* essential oil, the reduction rate decreased gradually from 40% the first day to 12.587% the tenth day. At concentrations of 1100 ppm and 2500 ppm, the reduction rate remains growing during the ten days of experimentation and pass from 70% the first day to 91.608% the tenth day. No growth of *Penicillium camembertii* mycelia was observed at the three tested concentrations of *Cymbopogon citratus* essential oil. The mycelial reduction rate was increasing and began from 0% the first day to 92.2% the last day. After having reintroduced the mycelial disks of the plate where no growth was observed (*Cymbopogon citratus* at 1100 ppm, for *Aspergillus niger* and *Fusarium oxysporum*, at 500 ppm for *Penicillium camembertii*) on an agar medium without essential oil, no resumption of mycelial growth was observed. These results could be explained by a fungicidal effect of this volatile extract.

Table-1
Chemical composition of essential oil extracted from the leaves of *Cymbopogon citratus*

Components	**KI**	**%**
6-methyl-hep-5-en-2-one	985	1,2
Myrcene	**991**	**10,4**
Limonene	1031	-
(Z)-β-ocimene	1036	0,2
(E)-β-ocimene	1047	0,2
6,7-epoxymyrcene	1091	0,2
Pirillene	1098	0,1
Linallol	1100	0,5
2,2-octa-3,4-dienal	1106	0,1
cis-vervenol	1140	0,1
trans-verbenol	1144	-
menth-3-en-9-ol	1150	0,1
Citronella	1153	0,4
cis-chrysanthenol	1162	0,5
epoxy rose furane	1170	0,2
Nerol	1231	0,3
Neral	**1245**	**33,0**
Geraniol	**1256**	**6,6**
Geranial	**1276**	**41,3**
neryl formate	1285	0,1
geranyl formate	1299	-
géranyl acetate	**1378**	**2,4**
β-caryophyllene	1419	-
trans-α-bergamotene	1435	-
Caryophyllene oxide	1587	0,1
Vulgarone	1659	-
Oxygenated aliphatic compound		**1,3**
Oxygenated monoterpens		**85,5**
Hydrogenated Sesquiterpens		**-**
Oxygenated Sesquiterpens		**0,2**
Total		**98,1**

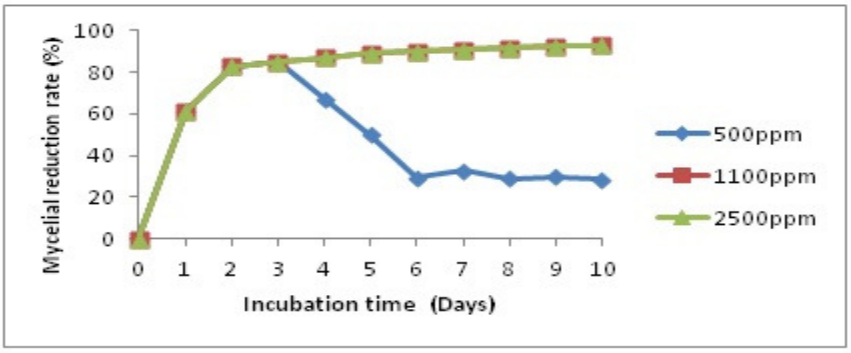

Figure -2
Reduction of mycelial growth of *Aspergillus niger* based on various concentrations of *Cymbopogon citratus* essential oil

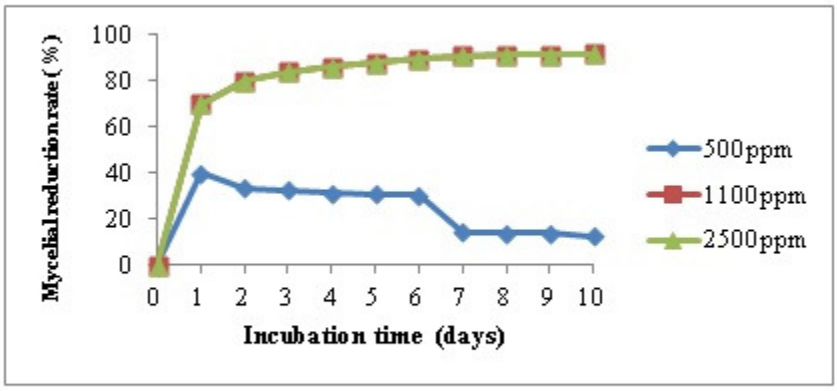

Figure -3
Reduction of mycelial growth of *Fusarium oxysporum* based on various concentrations of *Cymbopogon citratus* the essential oil

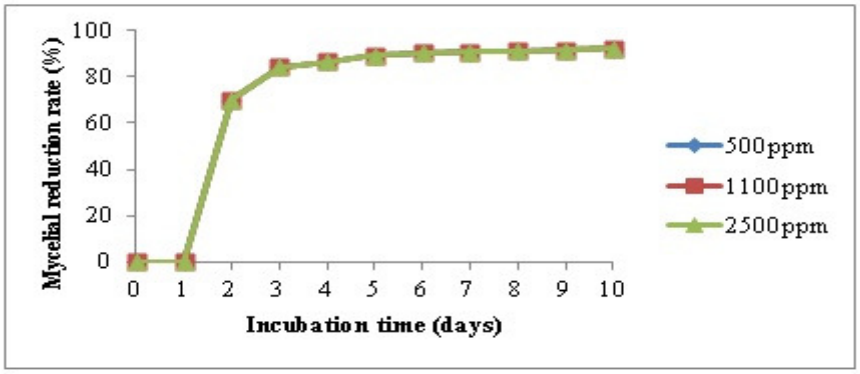

Figure 4
Reduction of mycelial growth of *Penicillium camembertii* based on different concentrations of *Cymbopogon citratus* essential oil

The minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) of *Cymbopogon citratus* essential oil on *Saccharomyces cerevisiae* were 0.24 mg/ml and 0.48 mg/ml respectively. This oil possessed antifungal power (fungicidal activity) against *Saccharomyces cerevisiae*.

Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC): The table 2 indicates the minimal inhibitory and bactericidal concentrations of the volatile extract on the two bacterial strains *Escherichia coli ATCC 25922* and *Staphylococcus aureus ATCC 25923*. Essential oil of *Cymbopogon citratus* possessed bactericidal activity against the two bacteria tested with MIC equal to 0.98 mg/ml and 0.48 mg/ml respectively on *Escherichia coli* ATCC

25922 and *Staphylococcus aureus* ATCC 25923. MBC of this oil on these two strains were respectively 1.98 mg/ml and 0.98 mg/ml. These results confirmed once again the antimicrobial activity of this oil underlined in literature by Chaumont *et al.* [29] and Koba *et al.*[30].

Table-2
Minimal Inhibitory and Bactericidal Concentrations of the studied volatile extract

Plant / Strains	Parameters	*Cymbopogon citratus*	Nature of activity of essential oil on strain tested
***Escherichia coli* ATCC 25922**	MIC (mg/ml)	0.99	
	MBC (mg/ml)	1.98	Bactericidal
	MBC/MIC	2	
Staphylococcus aureus* ATCC *25923	MIC (mg/ml)	0.49	
	MBC (mg/ml)	0.98	Bactericidal
	MBC/MIC	2	

Essential oil action on Tchakplalo stabilization: The analysis of the table-3 shows that the samples presented humidity rates varying between 86% and 88% and the dry matter rates variable from 12% to 14%. The sample E1 present the highest °Brix values as well as rate of acidity (14.8 and 6.43g/l respectively). The E2 sample which has received the essential oil of *Cymbopogon citratus* showed total soluble dry matter rate (MSST or ° Brix) of 14,6. The rate of acidity of the samples varied from 5.07 to 6.37 g/l. All samples presented an alcohol rate lower to 6% in volume. The E1 sample (positive witness) had the highest sugar totals rate (4.19%) followed by the E2 sample which has received the essential oil of *Cymbopogon citratus* (3.30%). The E0 sample (negative witness) presented the weakness sugar rate (2.83%). These results are in accordance with the results obtained by Aka *et al.* [31] who studied the variability of the physicochemical and microbiological properties of the tchapalo appropriated in Abobo, township situated in the North-East of Abidjan. It is evident from this survey that the quality of musts and tchapalo products was constant. The pH, sugars content, Brix value and acidity of this drink were 3.4, 3.6g/100, 7.9 and 5.2 respectively. The tchapalo obtained after fermentation contained 5.2% of ethanol on average. Lyumugabe *et al.* [32] had similar results while doing a physicochemical characterization of the Ikigage, a Rwandan traditional beer based on sorghum with regard to the pH (3.9), Brix value (11.6); they however observed weaker values of acidity (1.72 g/l) and alcohol rate (2.2 g/l). Sawadogo *et al.*[33] found also weaker values of the titrable acidity (0.13% to 0.61%) during their survey on the predominant lactic bacterium biodiversity in the dolo and the pito, two traditional beers based on sorghum respectively of Burkina and Ghana. All our samples of tchakpalo presented a weak alcohol rate (<6% flight). This result could be explained by the weak content in fermentable sugars of these drinks. According to Novellie [34] it can be the consequence of a weak content of α -amylase. The weakness of the total sugar rate in the negative witness sample E0 could be explained by a salvage fermentation of sugars due to the presence of yeasts in this sample. The E1 sample (positive witness) seemed to be the most stable with an average of 6 cfu/ml of total bacteria during the the storage followed by the samples which has received the essential oil of *Cymbopogon citratus* with an average of 22 cfu/ml of total bacteria counted during the storage. The analysis of the figures 5 and 6 showed a presence of the yeasts as well as an increasing evolution of the total bacteria (from 95 cfu/ml the first week of storage to 250 cfu/ml at the tenth week) in the negative witness E0. The presence of yeasts and total bacteria in the E0 sample could be explained by the non stabilization of the product. The only operation of pasteurization could not eliminate all microorganisms therefore. Indeed, according to Moll and Torija [35], during fermentation, the yeasts use the fermentable sugars that they transform in alcohol and in dioxide of carbon. At the time of the brewing, the starch is partially hydrolysed by the amylases. This important hydrolysis of sugars would be the proof of the presence in the ferment of producers' microorganisms of hydrolase. The stabilization of the two (02) other samples (E1, E2,) respectively by *Cymbopogon citratus* essential oil and citric acid before the pasteurization could be explained by the absence of moulds and bacteria in these samples; the antifungal and antibacterial activities of these preservatives which have been mentioned above. Mouara *et al.* [36] observed that on must obtained after heating, the yeasts are practically absent safe in the "bili bili" where they were brought by the inoculum used to accelerate fermentation.

Table- 3
Results of physico-chemical analysis of product tchakpalo

Parameters	Samples		
	E_0	E_1	E_2
Humidity (%)	88 ± 0.41a	86± 0.41b	87 ± 0.41c
Dry matter (%)	12 ± 0.41	14 ± 0.41	13 ± 0.41
Brix	15.2 ±1.53	14.8 ±1.53	14.6 ±1.53
pH	4.3 ±0.46	4.2 ±0.46	4.4 ±0.46
Acidity (g/l)	6.37 ±0.41	6.43 ±0.41	5.07 ±0.41
Ethanol (% v/v)	**< 6**	**< 6**	**< 6**
Total sugars	2.83 ±0.78	4.19 ±0.78	3.26 ±0.78

Eo = Sample witness, E1 = Sample having received citric acid, E2 = Sample having received the essential oil of *Cymbopogon citratus*.

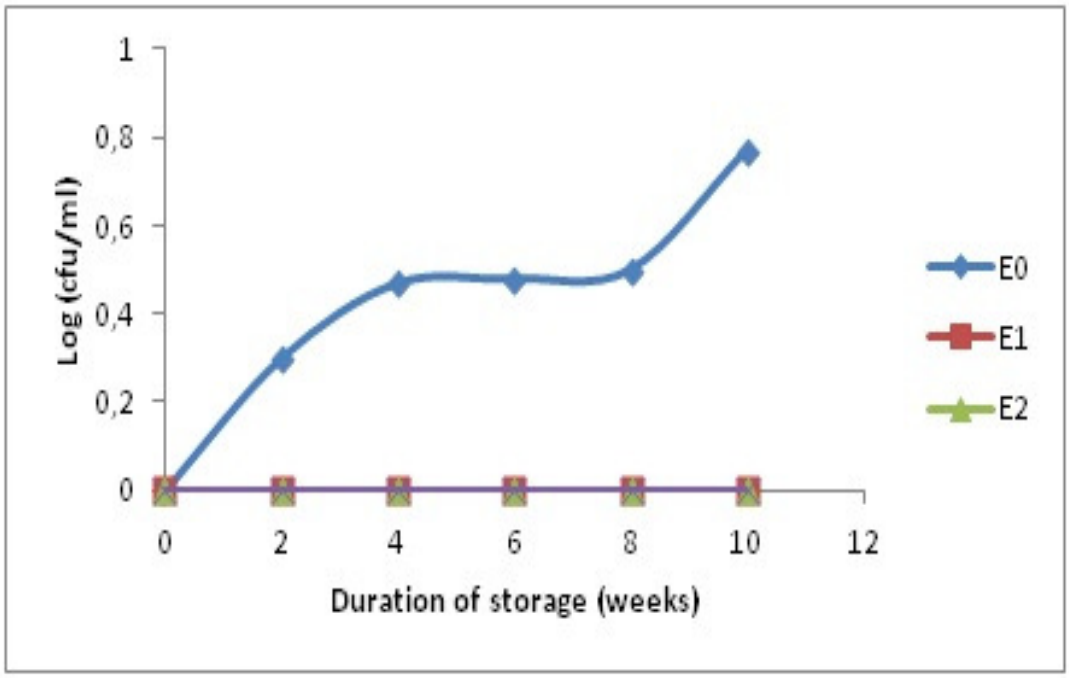

Figure -5
Evolution of yeasts in the different samples during storage

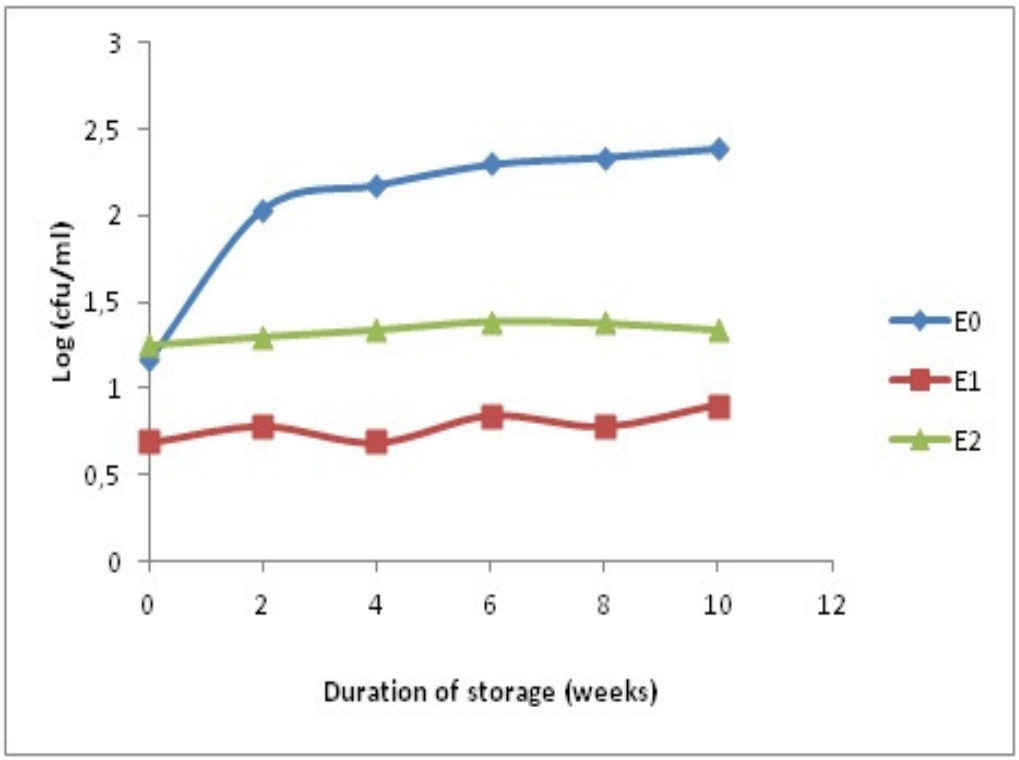

Figure -6
Evolution of total aerobic mesophilic bacteria in different samples during storage
Eo = Witness sample E1 = Sample with citric acid; E2 = Sample with essential oil of *Cymbopogon citratus*.

Sensory evaluation of Tchakpalo stabilized: Figures 7 and 8 present the results of the evaluation of sensory quality of different samples of tchakpalo. It was evident from these results that with regard to the taste and flavor, all panellists think that all samples have a pleasant or very pleasant taste.

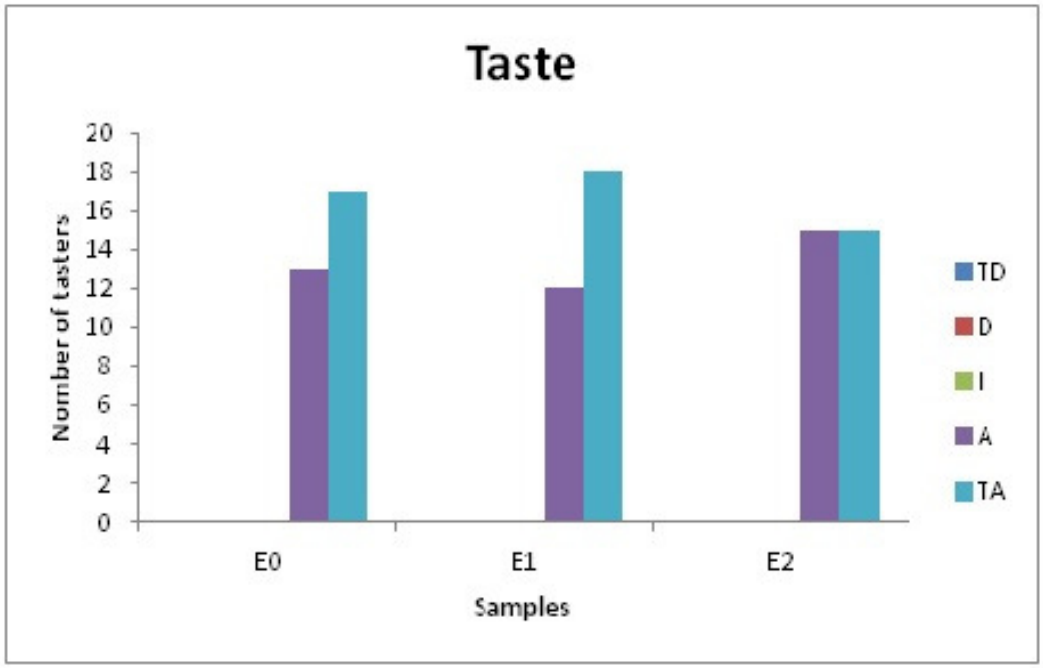

Figure -7
Organoleptic appreciation (taste) of samples of tchakpalo

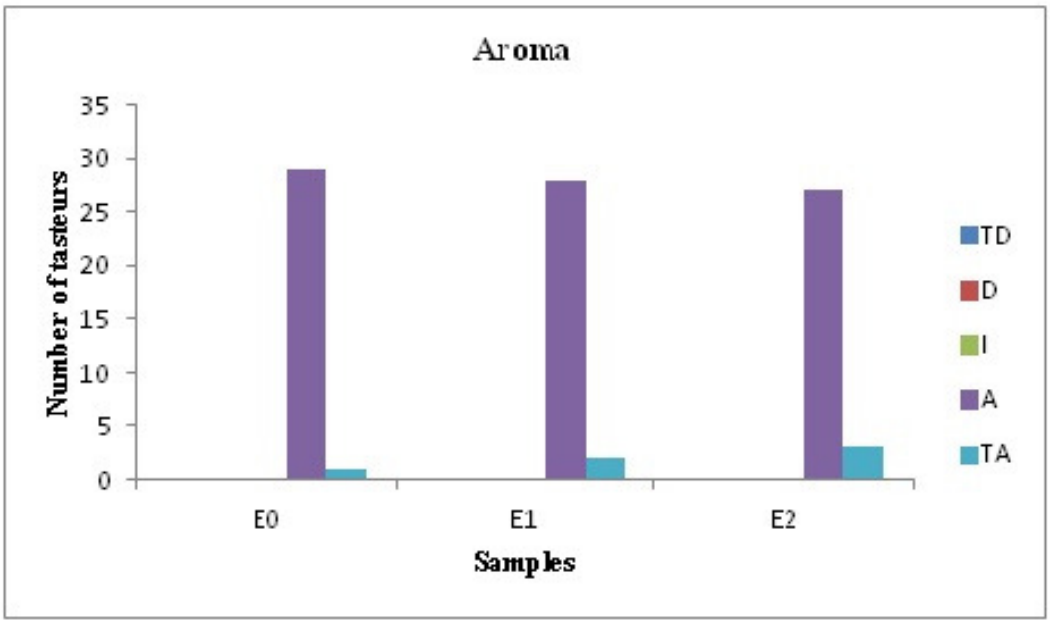

Figure- 8
Appreciation organoleptic (flavor) samples tchakpalo
Eo = Witness sample E1 = Sample with citric acid; E2 = Sample with essential oil of *Cymbopogon citratus*.
TD = very unpleasant, D = unpleasant, I = Indifferent A = Pleasant, TA = Very Pleasant

Conclusion

The rich and varied Beninese flora has numerous aromatic species reputed for their antimicrobial and foods preservatives properties. The present work permitted to put in evidence the conservative power of essential oil of *Cymbopogon citratus* harvested for the preservation of the traditional beer Tchakpalo, a local drink based on sorghum in Benin.The results obtained from this work reveal that the essential oil of *Cymbopogon citratus* possessed high antimicrobial properties against spoilage (*Saccharomyces cerevisiae*, *Penicillium camembertii*) and pathogenic microorganisms (*Aspergillus niger, Fusarium oxysporum, Escherichia coli ATCC 25922* and *Staphylococcus aureus* ATCC 25923) isolated from this drink or susceptible to contaminate it. The essential oil of *Cymbopogon citratus* in addition to Tchakpalo permitted to make steady this drink during ten weeks. Even though, the conservative power of this essential oil remained was weaker than the one of the citric acid in this survey. Its use for the conservation of foods must be counseled in order to limit the risks of poisonings due to the chemical additives usually applied for conservation.

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