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Efficiency of the essential oil of *Mentha spicata* L. in the stabilization of sorghum beer "tchakpalo" produced in Benin

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ABSTRACT

Several synthetic chemicals used as preservative of food crops have been banned in some countries because of their toxicological effects (adverse reactions). This study aims the use of plant extracts as natural preservatives of a sorghum beer "*tchakpalo*". The drink has been produced following the traditional technology improved by the addition of the *Mentha spicata* L. essential oil. This essential oil was extracted by hydrodistillation with a Clevenger type apparatus and analyzed by gas chromatography and by gas chromatography coupled to mass spectrometry. The antifungal activity of this oil was evaluated by the method of diffusion in agar against the strains responsible of the alteration (*Aspergillus flavus*, *Aspergillus parasiticus* and *Penicillium digitatum*) isolated from "*tchakpalo*". The extraction yield of essential oil was 0.27% after 135 minutes. The major compounds identified in this essential oil were: carvone (66.57%), limonene (16.33%) and 1.8-cineole (7.22%). Antifungal activity evaluation showed fungicidal effect of this essential oil on *A. flavus* at a concentration of 0.27 mg/ml and has shown fungistatic effect on the other two fungi at the concentrations tested. The addition of this essential oil at 0.05 % in the beverage produced has made it stable during two (02) months. It would therefore be advisable to continue the studies on a longer "shelf life" in order to confirm the conservative effect of this essential oil.

Introduction

In Africa, some cereal grains such as maize, millet and sorghum are often transformed in beverage whose manufacturing requires an essential step of alcoholic fermentation [1]. In Benin, sorghum is usually transformed into traditional beers such as "Tchoukoutou" and "Tchakpalo". This latter is often solicited during events such as baptisms, funerals, marriages and traditional festivals [2]. However, the tchakpalo is a favorable environment for the development of micro-organisms which impair its satisfactory and health qualities. Because of the influence of the germs, it becomes unstable and must therefore be consumed immediately after its production, otherwise the fermentation continues and it becomes sour and inconsumable. The difficulties of its preservation and problems related to its hygienic quality limit its valuation [3]. Despite the improvement of food preservation techniques, the nature of the food preservatives remains one of the most important problems for the health of consumers [4]. To cope with foodstuffs oxidation and contamination, the development of chemistry has fostered the emergence and application of new chemical substances such as benzoates, sulphites, calcium chloride, citric acid etc as food preservatives [5]. Subsequently, several of these chemical preservatives have been banned in many countries because of their toxicological effects adverse long-term, including carcinogenicity

The current trend of consumers seeking a healthier diet is the research food natural products with antimicrobial activities such as plant extracts. These extracts, reservoirs of natural antioxidants, also have antimicrobial activities and are without major effects on the environment and human health [4, 7]. More recently, other authors have reported the effectiveness of plant extracts in the conservation of the tomato [8], groundnut [9], the fulani cheese "waragashi" [10], fish [11], margarine [12], the meat [13]. These leaves are often used in food and aromatherapy because of the beneficial effect of their aromas on human health. Although the antimicrobial properties of some essential oils are studied, their applications as natural preservative in food are very little developed. However, the use of essential oils for the stabilization of the tchakpalo could be an alternative more integrated. The objective of this work is to study the antimicrobial activity of the essential oil extracted from M. spicata L. leaves and to assess its effectiveness in the stabilization of local drink "tchakpalo" of their toxicological effects, including carcinogenicity [6].

Materials and methods

Collection of the plant material

The grain of red sorghum (*Sorghum bicolor* L.) Moench) used for the production have been purchased in Dantokpa market (Latitude/Longitude: 6.37/2.43) at Cotonou in Southern Benin. The leaves of *M. spicata* L. were harvested at *Cadjehoun* (Cotonou) on a sandy soil and essential oil of this plant was extracted by distillation using an apparatus type Clevenger. This oil was dried using sulfate anhydrous sodium and stored at + 4°C.

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Study of the chemical composition of the essential oil

The analysis of the chemical composition of the essential oil was performed by using gas chromatography (type VARIAN CHROMPACK - CP 3800) by injection of 0.2 µl of extract. The carrier gas used is Helium (He) with a flow rate of 0.3 ml/minute. The column used is a capillary column type CP-Chirasil-Dex CB fused silica WCOT, 25 m length and 0.25 mm internal diameter. The thickness of the stationary phase is 0.25 µm; programming temperature of the initial injection column is 70ºC for 2.50 minutes, and then rises by steps of 15 °C/min to 240ºC for 20 minutes. The detector used for this analysis is FID type with 250ºC as temperature. The device is controlled by a computer with analysis software and a data bank and a NIST database which allows the identification of compounds.

Isolation and identification of fungi.

Aliquots of 0.1 ml of the drink taken after 48h of fermentation and their decimal dilutions (10^{-1}, 10^{-2} and 10^{-3}) were seeded on the surface on agar Sabouraud + Chloramphenicol, originally prepared and poured in Petri dishes of 9cm in diameter. The enumeration of moulds which occur in a powdery form was performed after 5 days of incubation at 25°C according to standard NF V08-059 [14]. These analyses were repeated by seeding on the media Potato Dextrose Agar (PDA) and Yeast Extract Agar to confirm the germs isolated during the identifications. Each colony of mould obtained has been isolated from yeast, purified, identified and reisoled by touches in new Petri dish on agar Sabouraud + Chloramphenicol and incubated at 25°C until complete purification of the strains. The purified stem have been isolated by key on both identification medium Malt Extract Agar and Czapeck Yeast autolysate Agar prior to their identification based on their growth characteristics, macroscopic and microscopic according to the keys of Samson *et al* [15], Pitt and Hocking [16].

In vitro antifungal activity of the essential oil

Evaluation of antifungal activity of essential oils on moulds isolated from the *tchakpalo* was performed following the method reported by Fandohan *et al* [17]. Moulds were transplanted with a disc (washer) of 6 mm diameter on the culture medium containing the essential oil at different concentrations (0, 100, 200 and 300 mg/ml) pre-poured in Petri-dishes. These boxes were incubated at 25°C and the mycelia growth was measured every 24 hours by measuring the average of two perpendicular diameters passing through the middle of the washer during seven (07) days. The antifungal activity was assessed by the following formula: I= [1-(d/dc)] x100 [18] With I: antifungal index; d: diameter of growth of the box treated with the essential oil; dc: diameter of growth of the control (witness) [box without essential oil]. For each concentration, 3 recurrences were performed.

Process of *tchakpalo* production

The production of the local drink *"tchakpalo*" has been made according to the traditional process used by producers and reported by Konfo *et al* [19]. It includes three main steps (malting, brewing and fermentation).

The malting

Malting process of sorghum grain shows two steps

- **Cleaning and soaking**: Sorghum was getting rid of the various impurities (rotten grain or broken, weed seeds, other plant debris, other waste) by winnowing, sorting and washing. The clean and wholesome grains were then placed in a container containing a large quantity of cold water. After three days, they were removed from the water, rinsed and set to drain.

- **Germination and drying**: The grains well drained were spread on a jute bag in a thin mat of 4 to 5 cm thick and were covered with another jute bag. This is germination process, which lasts three days. The grains were then dried under sun during three days.

Brewing

- **Crushing, mashing and soaking 1**: the malt was crushed and the coarse flour obtained was mixed with water and kneaded until the moderately consistent batter. This paste was left in the sun for seven hours before being dissolved in water (2 to 3 liters of water for 1 kg of grain). The suspension thus obtained was homogenized, and then left at rest at room temperature (28-29°C) for eight hours.

- **Cooking 1**: the mixture separates into two phases (decantate 1, and decantation residue 1). The residue was collected and melted in a large amount of water (3 liters for 1 kg of grain). The whole is brought to a boil. The duration of the cooking time was 2 hours and 30 minutes at 106°C.

- **Soaking 2**: the baked product thus obtained was mixed with the supernatant previously set aside (decantate 1) and the whole was abandoned for a dozen hours.

- **Cooking 2**: The cooled mixture separates into a supernatant (decantate 2) and the deposition (residue of decantation 2). This last (the decantation residue 2) was collected, diluted in water (1 to 2L. for 1 kg of beans) and cooked until the temperature of 95°C. at 45 minutes before being cooled and filtered. The filtrate was mixed with the decantate 2 to obtain the initial wort sterilized during 5 hours at 102°C.

Fermentation and filtration

The final wort was filtered and filtrate fermented for two (02) days. After this phase of fermentation, drink was filtrated to remove some of the particles in suspension.

This traditional technology has been modified by addition of 0.05% of the *M. spicata L* essential oil., during conditioning and then, the samples were stabilized by pasteurization. The control samples (simply pasteurized) have also been produced. Other witnesses have received the same dose of citric acid (used as a standard preservative). Figure 1 presents the diagram of *tchakpalo* production.

Physico-chemical characterization of samples products

The pH was measured with a pH meter (HANNA HI 98129) and the Brix was determined using a refractometer (ATAGO, Japan). The tritritable acidity expressed as citric acid percentage per unit of volume was determined by dosing with sodium hydroxide 0.1 mol/L in the presence of phenolphthalein as indicator [20]. Total sugars, alcohol, and ash were determined respectively according to method reported by Dubois *et al* [21] , using alcoholmeter and by the AOAC [22]method.

Evaluation of the microbiological quality of samples during conservation

The evaluation of *M. spicata L*. essential oils effectiveness in *tchakpalo* conservation was made by microbiological analysis performed during two (02) months after storage. Samples were evaluated by using microbiological standard methods. These were the total aerobic mesophilic flora on the Plate Count Agar (PCA) middle and incubated at 30ºC for 24 h [23], total coliforms and thermotolerant on the Violet Red Bile Lactose (VRBL) middle with incubation at 30 and 44ºC respectively, for 24 h [24], *Staphylococcus aureus*, on Baird Parker media and

*Related to improved process
E O = essential oil

Figure 1. Manufacturing process of Tchakpalo.

incubation at 37°C for 48hours [25], yeasts and moulds on Yeast Extract Sucrose media and incubated at 25 °C for 5 days [14].

Organoleptic analyses

This assessment has focused on the aroma and the taste of the drink. It has been carried out through a panel of thirty tasters interviewed on-site marketing.

Statistical Analyses

The results obtained were subjected to analysis of variance and the averages from three repetitions were separated by Tukey multiple comparison test with the statistical software SPSS 16.0. Differences with a probability value less than 0.05 ($P<0.05$) are considered significant.

Results

Extraction yield of the essential oil

The yield of essential oil from *M. spicata L.* is 0.27% (figure 2) after one hour thirty minutes of extraction.

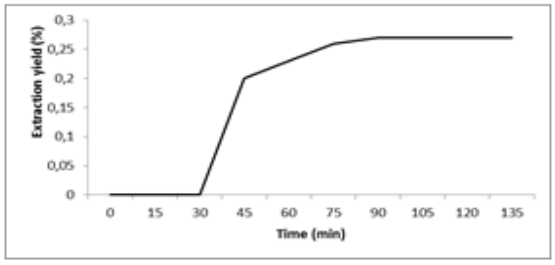


Figure 2. Evolution of the extraction yield of *M. spicata* essential oil in the time.

Chemical composition of essential oil

The table 1 presents the chemical composition of the essential oil of *M. spicata L.* The analysis of this table shows that majors compound in *M. spicata L.* essential oil were carvone (66.57%), limonene (16.33%) and 1.8-cineole (7.22%).

Table 1. Chemical composition of the essential oil *of M. spicata*.

Compounds name	KI	(%)
α-Pinene	932	0.94
Sabinene	969	0.51
β-Pinene	974	1.55
Myrcene	988	0.59
Limonene	1024	16.33
1.8-cineole	1026	7.22
8-terpinene	1054	0.1
Linalol	1095	0.06
δ-terpineol	1166	0.1
Carvone	1239	66.57
Pipéritone	1249	2.49
α-copaene	1374	0.2
(Z)-caryophyllene	1408	0.8
α-humulene	1454	0.1
Germacrene-D	1484	0.4
Cis-calamenene	1528	0.9
α-cadinol	1652	0.5
Not identify		0.5
Total		99.86

Moulds identified and *in vitro* antifungal activities of the essential oil of *M. spicata* L.

The microbiological analyses carried out on the beverages sampled after fermentation were used to isolate and identify three fungi strains. There were: *A. flavus*, *A. parasiticus* and *P. digitatum*. Figures 3, 4 and 5 show the reduction rate of the mycelial growth by different concentrations of essential oil on these strains. It should be noted that the increase in reduction rate reflects the inhibition of the mycelial growth, while its decrease expresses the mycelial growth of the mould tested. At 300 mg/ml, visible growth of *A. flavus* has not been observed during the seven days. The reduction of this mould growth at 200 mg/ml increases from 73.07% on the first day to 76.36% the seventh day. At 100 mg/ml, there was a decrease in the rate from 61.53% the first day to 18% the fourth, followed by an increase to 30.90% the seventh day. The higher discount rate on *A. parasiticus* was 80.83% at 300mg/ml and the lowest obtained was 37% at 200mg/ml of essential oil. The inhibition rate obtained on *P. digitatum*, oscillated between 10% and 100%. The essential oil of *M. spicata L.* appeared to be most active on this strain at 300 mg/ml.

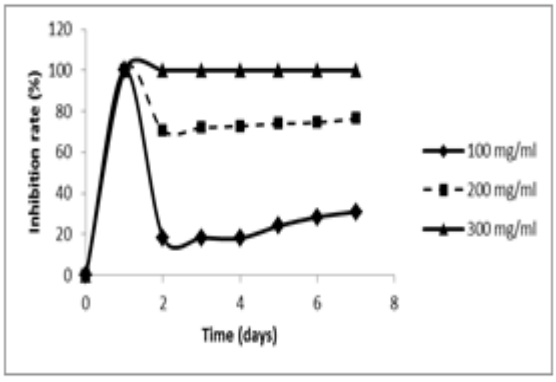


Figure 3.Reduction rate of *A. flavus* depending on time.

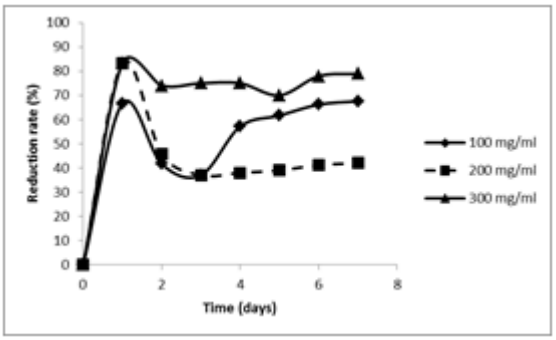


Figure 4. Reduction rate of A. parasiticus depending on time.

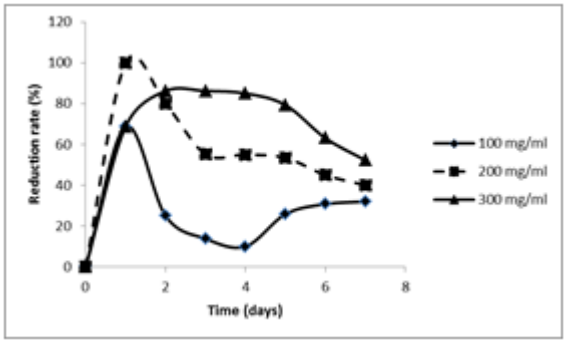


Figure 5.Reduction rate of P. digitatum depending on time.

Physico-chemical characteristics of *tchakpalo* samples

The physico-chemical characterization of the samples (table1) shows values of pH, acidity titratable and Brix ranging respectively from 3.18 ± 0.27 to 3.22 ± 0.16 ; 4.73 ± 0.39 to 5.31 ± 0.00 and 1.93 ± 0.25 to 2.03 ± 0.16.The lowest content of total sugars was also observed in pasteurized samples (3.36±0.04). The highest total sugars content (4.03±0.04) was obtained for samples that received citric acid. These values were inversely proportional to the alcohol content and vary from 1.00±0.00 and 1.5±0.00 for the same samples. Ash content of the samples varied from 9.93 ± 0.28 to 12.50 ± 1.68.

Table 2. Physico-chemical characteristics of samples of tchakpalo.

Parameters / Samples	E_1	E_2	E_3
pH	3 .18 ± 0.27[a]	3.22 ± 0.16[a]	3.21 ± 0.17[a]
Titratable acidity (g/L)	4.73 ± 0.39 [a]	5.31 ± 0.00 [a]	5.26 ± 0.34 [b]
Brix	1.93 ± 0.25 [a]	2.03 ± 0.15 [a]	2.03 ± 0.16 [a]
Total sugars g/100g)	3.36 ± 0.04 [a]	3.95 ± 0.05[b]	4.03 ± 0.04 [b]
Alcohol content (% vol)	1.50 ± 0.00 [a]	1.00 ± 0.00 [a]	1.00 ± 0.00 [a]
Water content (%)	90.07±2.75 [a]	87.50±1.68 [a]	87.80± 1.53 [a]
Dry matter (%)	9.93 ± 0.28 [a]	12.50±1.68 [a]	12.20± 1.53 [a]

The means followed by same letter in the same line are not significantly different at 5%.

E_1: Pasterized beverage without preservative

E_2: Pasterized beverage + essential oil of *M. spicata*

E_3: Pasterized beverage + citric acid.

Evaluation of the conservative effect of *M. spicata* L essential oil

Table 3 presents the evolution of the microbial flora in *tchakpalo* during the storage.

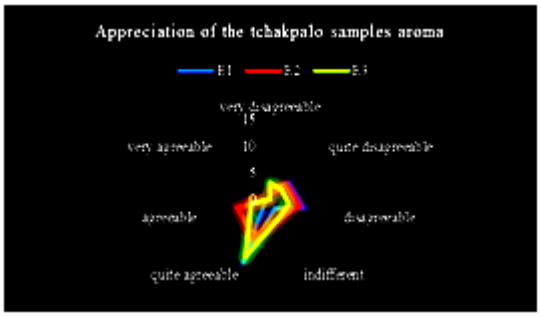
Table 3. Microbiological changes during *tchakpalo* storage.

Germs researched	Samples	Storage duration (days)		
		0	30	60
Total flora (CFU/mL)	E_0	117	234	352
	E_1	86	124	207
	E_2	26	72	96
Yeasts (CFU/mL)	E_0	96	146	174
	E_1	53	79	105
	E_2	<1	02	03
Moulds (CFU/mL)	E_0	<1	05	07
	E_1	<1	<1	<1
	E_2	<1	<1	<1
Total coliforms (CFU/mL)	E_0	<1	<1	<1
	E_1	<1	<1	<1
	E_2	<1	<1	<1
Thermotolerants coliforms CFU/mL)	E_0	<1	<1	<1
	E_1	<1	<1	<1
	E_2	<1	<1	<1
Staphylococcus aureus (CFU/mL)	E_0	<1	<1	<1
	E_1	<1	<1	<1
	E_2	<1	<1	<1

This table reveals the presence of total flora in all the samples. However, their number was raised to the level of controls stabilized by pasteurization than those who received preservative before this operation. These same observations were made for yeast and mould.

Organoleptic characteristics of beverages

The results of the sensorial characterization revealed that 53.33% of the tasters thought that the E_3 aroma was pretty nice. 43.33% thought that E_1 was quite agreeable, and 26.66% for E_2. In the taste, 93.33% find E_0 not sweet. This point of view is shared by 86.66% of the tasters on E_2.



E_1: Pasterized beverage without preservative

E_2: Pasterized beverage + essential oil of *M. spicata*

E_3: Pasterized beverage + citric acid

Figure 6. Appreciation of the *tchakpalo* samples aroma.

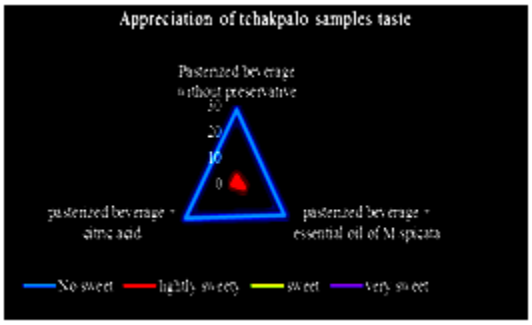


Figure 7.Appreciation of *tchakpalo* samples taste.

Discussion

The yield of essential oil of the leaf of *M. spicata L.* increase in time and reach an optimum 0.27% after 90 minutes

of heating. This extraction yield is substantially lower than that obtained in 2012 by Adjou and Soumanou [9], which was 0.96% for the same plant harvested to *Cadjehoun* (Cotonou) on a ferralitic soil sandy. Znini *et al* [26] have obtained a yield that approximates the double of our (0.53%) in South-east of Morocco. The identification of the chemical compounds of the extracted oil showed the presence of carvone, limonene and 1.8 cineole as major compounds. This chemotype is different from those reported by Tayarani-Najaran *et al* [27] in Iran, rich in pulegone (56.28%), α-terpineol (8.75%), limonene (6.35%), isomenthone (5.75%) and enlongifolène (5.47%). Hadjiakhoondi *et al* [28] have found as compounds in the majority for these same leaves the carvone (22.40%), linalool (11.25%) and limonene (10.80%) in the same country. This chemotype is also different from that reported in Morocco by Znini *et al* [26] which were carvone (29%), trans-Carveol (14%), 1.8-Cineol and limonene (7%). These differences observed at the level of the yields and the chemical composition could be related to the area of collection, the nature of the soil, the harvesting period and the stage of plant development [9]. The analysis of the results from the evaluation of the effect of the essential oil on the fungal strains isolated from the beverage reveals that this oil activity is more pronounced on *A. flavus* than the other two strains, with a reduction rate minimum 13.79% at 100 mg/ml and total inhibition at 300mg/ml during the seven days. This oil holds, therefore, at the concentrations tested fungicide effect on *A. flavus* and a fungistatic effect on *A. parasiticus* and *P. digitatum*. The antifungal activity of this plant species would be due to the chemical composition of its essential oil. Indeed, Bansod *et al* [29] reveal that the antimicrobial activity of the essential oil of *M. spicata L.* against Aspergilla, particularly the species *niger* and f*umigatus* would be due to compounds in the majority: the Carvone (49.5%) and Menthone (21.9%). These compounds, which could act alone or in synergy with the compounds in the minority, would have a mode of inhibition action of spore germination, elongation of the mycelium, sporulation and production of toxins in these moulds. The physico-chemical characterization of the samples shows that there are no significant differences ($P > 0.05$) in the level of pH, Brix, moisture content, ash and alcohol content of different samples. There is also no significant difference compared to the sugars rate between the samples stabilized with the addition of preservatives (essential oil or citric acid). However, a difference of these parameters was observed in the sample stabilized by pasteurization ($P<0.05$). The high value of the acidity of the samples of batch E_3 could be explained by the addition of citric acid as a preservative. This could also justify the results obtained for the high rate of sugar in the same sample, as the acidification of the environment would inhibit the activity of the yeast at the origin of the consumption of sugar. The high content of alcohol at the level of the stabilized samples by simple pasteurization could be due to a fermentation residual which is the lack of substance with antifungal properties consequences. These results are in agreement with those obtained by Aka *et al* [30] for their work on the variability of physico-chemical properties during the alcoholic fermentation of the samples of sweet wort and *tchapalo* collected in an area of mass production in *Abobo*, a commune located at the North-east of Abidjan (Ivoiry Coast). Lyumugabe *et al* [31] have found similar values by performing a physico-chemical characterization of the *ikigage* in Rwanda, in relation to the pH (3.9) the Brix (11.6). However, these same authors obtained lower values for the titratable acidity (1.72 g/l) and relatively stronger for the alcohol rate (2.2%). The results of the evaluation of the stability of the microbiological samples as a function of time confirm those obtains in vitro. Indeed, the elimination of all mould as well as the decrease in the number of colonies of the total aerobic mesophilic flora and yeast in the samples that received the essential oil would be due to the stabilizing properties due to the antifungal activity of this oil. Thus, the use of plant extracts (natural products) as an effective alternative then becomes an emergency. In fact, the studies done around the world show that the essential oils can be added to almost all foods. The essential oils of oregano, thyme, cinnamon or coriander are effective for meat, poultry, pork, and vegetables; essential oil of mint for fresh products (salads, yogurt, etc.); essential oils of cavacrol or citral to the fish; the essential oils of thyme, nutmeg or ginger for cereals (particularly those rich in carvacrol for the rice); and essential oils of carvacrol or cinnamaldehyde to the fruit. However, some limits do exist to the use of essential oils as preservatives in the food, including flavours power of some of them. Nevertheless, desaromatization technics exist and are more efficient. On the other hand, the sensory effects can be limited by selecting carefully the essential oil according to the type of food considered, but it is important to note that in most cases, the concentrations of the oils used are so low, that they would not alter the organoleptic qualities of the food [32]. Of course these results are only a first step in research of antimicrobial products new and natural to offer in the agri-food industry. Additional testing must be able to confirm the performance highlighted. Knowing the toxicity of certain extracts, these tests must be supplemented by toxicity tests, primary skin, and tests of allergenicity.

Conclusion

The results from this work show that the essential oil of *M. spicata L.* has a variety of ways antifungal properties on the strains responsible of the alteration of the *tchakpalo* (*A. flavus, A. parasiticus and P. digitatum*). The addition of this oil to this drink helped to make it stable over a period of eight (08) weeks without a significant variation of the microbiological parameters with a concentration in order of 0.05%. There is also a better stability of the microbiological samples treated with citric acid compared to the other samples.

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