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RESEARCH ARTICLE

Antimicrobial activity of extracts from *Cymbopogon citratus* L. and of *Mentha spicata* L. against fungal and bacterial strains isolated from peuhl's cheese (Waragashi) produced in Benin

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Abstract

The present research aims to evaluate the antimicrobial potential of extracts from *Cymbopogon citratus* L. and *Mentha spicata* L. against the fungal and bacterial strains that cause peuhl's cheese (Waragashi) deterioration. The essential oils have been extracted by hydrodistillation with Clevenger-type apparatus and analyzed by gas chromatography and gas chromatography coupled with mass spectrometry. The non-volatile compounds have been extracted by maceration of the powder from leaves of each studied plants in aqueous solvents, ethanolic and hydroethanolic solvents. The antimicrobial properties of the extracts have been evaluated by the agar diffusion method against the strains isolated from cheese. The yields of extraction of essential oils were 1, 11% for *Cymbopogon citratus* and 0, 27% for *Mentha spicata*. The hydroethanolic extraction has most high yield for the two plants (18, 32 % and 15, 06% for *Cymbopogon citratus* and *Mentha spicata* respectively). The non-volatile extracts revealed the presence of catechic tannins, polyphenols and flavonoïdes in the two plants. The results from the microbiological characterization of the cheese samples reveal that they were all contaminated by mildews, yeasts, staphylococci, coliformes and *Escherichia coli*. The identification of the fungal flora of cheeses revealed presence of the Penicillium spp. During the tests, the volatile extracts were more active on the identified strains than their non volatile equivalents.

INTRODUCTION

The economy of the South Sahara African countries like Benin, is essentially based on agriculture and the breeding that have an important place in the economy (Diao et al., 2006). Among the products of breeding, the milk of cow has a large socio-economic importance. So, in Benin, the contribution of milk is more of 50% among the yearly incomes of the peuhl's households (Dossou et al., 2006). Milk is also transformed in various products drifted as yogurt, curdled milk and especially the waragashi, a cheese cooked according to an artisanal process mastered by the group ethnic Peuhl. The waragashi is the most widespread derivative product and the most used (Aïssi et al., 2009). This cheese constitutes an important source of animal proteins, mainly for the populations of weak incomes and could contribute validly to resolve some problems concerning the lack of proteins in the food of these populations (Keke et al., 2008). Several researches have taken place about its technology production, its microbiological quality and its conservation by the chemical additives, the thermal treatments as well as by strains of fermentation. In this logic, Aworth, (1985) and Kees, (1996) have studied the technology of production of the

waragashi, its stabilization by thermal treatment and chemical additive like the propionic acid and the sorbates of synthesis. But, the application of these chemical products of synthesis increases the probability of presence of toxic elements in the food (Moosavy et al., 2008). The consumption of these products can cause poisoning concerning chemical elements and its consequences that cannot be changed (cancers, resistance to the antibiotics, congenital malformation, obesity,…). Face to the frequency of these illnesses and indirect problems associated to consumers, the agro alimentary industry and the organisms of regulation on the use of some food additives of synthesis have imposed some limitations. Then, the importance of the use of natural alternative products becomes necessary (Bankole, 2004, Burt, 2004). That is why the interest for the natural products antimicrobial is increasing, in particular the vegetable extracts in the conservation of food (Hammer et al., 1999; Konfo et al., 2012). Among the vegetable plants *Cymbopogon citratus* and *Mentha spicata* are the most solicited for their antimicrobial properties and are also used in the food habits of the beninese population. Thus, the objective of the present research is to evaluate antimicrobial potential of the extracts (volatile and non volatile) of *Cymbopogon citratus* L. and of *Mentha spicata* L. against the strains of the destruction of the peuhl's cheese (Waragashi).

Material and Methods

Collection of the plant material

The used plant material is constituted of the fresh leaves of two food plants acclimatized in Benin. These leaves are *Mentha spicata* L. and of *Cymbopogon citratus* L. The collect has been made on sandy soil in the city of Cotonou (latitude/longitude: 6° 21' 45 " North / 2° 25' 32 " east) at the center market gardener of Cadjehoun. Concerning *Cymbopogon citratus*, it is collected in Abomey-calavi (latitude/longitude: 6°26' 54'' North/2° 21' 20'' east) on clayey soil. The plant material has been identified to the National herbarium of Benin.

Microbial strains tested

The microbial strains used for this research are essentially constituted of mildews (*Penicilluim*) isolated from the waragashi and the references strains of *Candida albicans* ATCC 10231 and of *Escherichia coli* ATCC 25922 obtained from the National Laboratory of the Ministry of the Public Health of Benin. The viability of these different strains of references has been verified by coloration of Gram before the use.

Obtaining of the extracts

The non volatile compounds have been obtained by maceration of the little pieces of the leaves dried respectively of the studied plant in ethanolic, hydroethanolique (V/V) and aqueous solvents. After filtration, the extracts are concentrated by evaporation at dry at 60°C with a rotavapor of Heidolph type. Essential oils extraction was made by hydrodistillation with Clevenger apparatus. The volatile extracts were recovered in a sterile amber small bottle of 10mL to limit the photochemical reactions. In the two cases, the output (R) % is calculated by the extracted mass (Me) obtained and the mass of the material plant introduced (Mv), according to the flowing formula:

$$R\ (\%) = (\mathbf{M}e/\mathbf{M}v) \times \mathbf{100}.$$

Composition chemical of the essential oils

Chemical composition of essential oils was determined by GC-MS: type Hewlett Packard-Quadrupôle (Model 5970). The chromatograph equipped with a capillary column of melted silica of 30m of length and interior diameter 0,25mm with dimehylpolysiloxane (HP5) of 0.25 m thick phase transplanted type. The temperature of the injector was fixed at 220°C, the voltage of the mass spectrometer was 70eV. The oven temperature was programmed at 70-200°C with a gradient of 10°C/min and the vector gas (helium) was adjusted to 0, 6 ml/min of debit.

Screening phytochimique of the extracts non volatile

The used procedure was based on reactions of colorations and precipitations more or less specific to every class of active principles (Table 1).

Table1. Specific reagents and reactions of the screening phytochimique

	Reagents	Observations	References
Tannins	$FeCl_3$ at 2%	coloration bruise-black or green black	Soro et al., 2009
Tannins cathechic	Reaction of STIASNY	Precipite of pink	Koudoro et al. 2014
Flavonoïdes	Reaction to the cyanidine	orange, red or purple coloration	Bruneton, 1999

Anthocyanes	Hydrochloric Acid diluted to 5%	red coloration that becomes more pronounced and turn to the purplish or greenish bruise	N'Guessan et al., 2009 ; Koudoro et al. 2014
Leucoanthocyanes	Hydrochloric Alcohol at 5%	coloration red cherry or purplish	Dohou et al., 2003 ; Rizk, 1982
Alkaloids	Reagent of Meyer and Dragendorff	apparition of a precipitate	
Derivative cyanogenic	Test of picric acid at 1%	Brown coloration	Bruneton, 1993 ; Dohou et al., 2003
Derivative quinonic	hydrochloric Acid at 5%	Pink Coloration to purplish red	Bekro et al., 2007
Saponines	Test of moss	Obstinate moss	Koudoro et al. 2014
Steroïdes	Test of Liebermann	Purple coloration change to the blue or to the green	Traore, 2010
Coumarines	Ammoniac at 25%	Intense fluorescence	Koudoro et al. 2014

Cheese Sampling

The sampling has been realized in three markets of Cotonou (South-Benin): the markets of Godomey, Dantokpa and Saint Michel. In every market, two cheeses small piece of 250±10 g has been taken by chance at three sellers. The withdrawal of the samples has been done in aseptic conditions: sterile gloves made of latex are used for the protection of the hands; the samples of cheese collected are wrapped in sterile sachets and are conditioned in an icebox. The different withdrawals of a same market have been mixed and have been broken in order to get a composite sample.

Microbiological analyses

In order to identify the strains which spoiled cheese, the different collected samples have been characterized. All manipulations have been done in conditions of total asepsis. Standard methods of microbiological analyses have been used. These analyses consisted in to the determination of the total flora at 30°C (NF V08-051), the total and thermotolerants coliformes (NF V08-050), staphylococci (NF EN ISO 6888-1/A1), the lactic bacteria (ISO 15214-1998), yeasts and mildews (NF V08-059, 2002). Mildews contaminating cheese have been isolated on Sabouraud with chloramphenicol and their identification has been made by microscopy based on the aspect of the bladders, of the metula, of the phialides and the conidies ((Filtenborg et al., (1995); Samson et al., (2004); Pitt et al., (2009)).

Antimicrobial tests

Antimicrobial activity evaluation of the extracts on mildews from cheese and the on bacteria has been perfommed according to method repported by Fandohan et al., (2001). It is proceed by the transplantation of mildews with the help of a disk (small disc) of 6 mm of diameter on space containing the extracts at different concentrations (100, 200 and 300 mg/ml) or no (witness) in the culture medium precast in Petri dishes. Dishes were incubated at 25°C and the mycelial growth has been measured after each 24H, measuring the average of two perpendicular diameters passing by the middle of the small disc during seven (07) days. The antifungal activity has been valued according to the following formula: I = [1-(d/dc)] x100 (Koudoro et al., 2000). With I: Inhibition rate, d: diameter of growth of dish treated with the essential oil; dc: diameter of growth of the control (witness). For every concentration, 3 repetitions were achieved on each of the isolated mildews.

Result and Discussion

Output of extraction of the essential oils

The yields obtained after the extraction of the essential oils from the two studied plants are presented by figure 1. Results showed that *Cymbopogon citratus* essential oil presents the most high extraction yield (1, 11%), against 0, 27% for *Mentha spicata*. The result obtained for *Cymbopogon citratus* essential oil was distinctly lower than value (1,70%) reported by Konfo et al., (2012) for the leaves of this same plant harvested in the plateau of Oueme (Benin) on sandy ferralitic soil. This yield was higher than this obtained by Kanko, (2010), on the same plant in Ivory Coast. and for Baba-Moussa et al., (2012) in Benin which was 0, 7%. Yield (0.27%) of *Mentha spicata* essential oil extraction was extensively lower to the one gotten by Adjou and Soumanou, (2013) that is of 0,96% for the same harvested plants in Cadjehoun (Cotonou) on sandy soil. Variability in these different yields would depend on the period of harvest, the season et also soil.

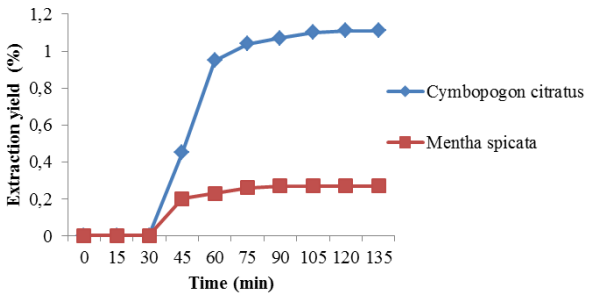

Figure 1: Extraction yield of essential oils during the times

Extraction yield of non-volatile compounds

The figure 2 indicated the extraction yield of the ethanolic, hydroethanolic and aqueous extract of the two studied plants. Hydroethanolic extract showed the higher yield (18.32% and 15. 06%), followed by aqueous extract (9.2% and 12%) and finally the one of ethanolic extract (7.7% 8.35%), respectively for *C. citratus* and *M. spicata*. It could be possible to say that the *C. citratus* chemical element has more affinity for the hydroethanolic extract than the chemical elements of *M. spicata*. What permits to say that *C. citratus* is richer in polar organic elements than *M. Spicata*. The chemical elements of *M. spicata* have more affinity for the aqueous and ethanolic extract than the chemical elements of *C. citratus*. This explains that *M. spicata* is richer in polar and organic elements than *C. citratus*.

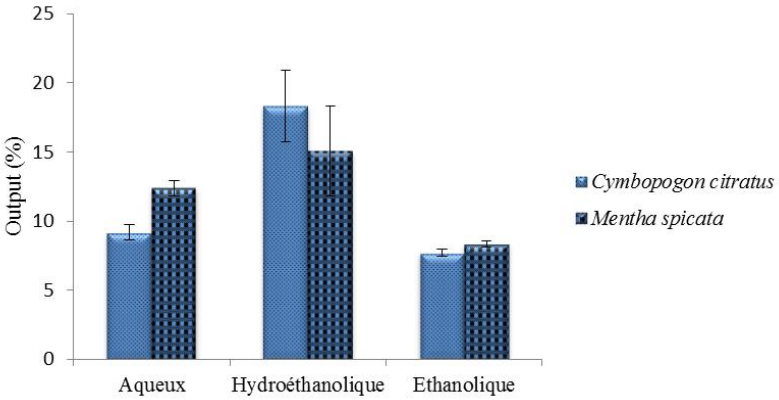

Figure 2: Non volatile extraction yield.

Chemical composition of the essential oils

The tables 2 and 3 presented respectively the chemical composition of *Cymbopogon citratus* and *Mentha spicata* essential oils. Table 2 reveals that the major components identified in essential oil of *Cymbopogon citratus* were: the geranial (41.3%), the neral (33%), the myrcene (10.4%) and the geraniol (6.6%). These results were similar to the ones of Koba et *al.*, (2004) in Togo; Nguefack et al., (2009), (2012) in Cameroon. These results were also in agreement with those of de Souza (1988), Yehouenou et al., (2010), Baba-Moussa et al., (2012) and Konfo et al., (2012) in Benin. It was noticed that *Cymbopogon citratus* essential oil has a chemical composition characterized by large presence of oxygenated monoterpenes like the neral, geraniol, and geranial. The oxygenated monoterpenes is in general antimicrobial elements with large spectre of activity (Alitonou, 2006). This confirms its high antimicrobial activity. Results (table 3) also show that the essential oil extracted from leaves of *Mentha spicata* was characterized by the presence of oxygenated monoterpenes (78.0%), hydrogenated monoterpenes (15.2%), hydrogenated sesquiterpenes (3.5%) and aromatic compounds (0.3%). The identified major components were carvone (66. 57%) and the limonene (16.33%). According to Tayarani-Najaran et al., (2013), the *Mentha spicata* leaves essential oil collected in Iran had as major compounds the pulegone (56.28%), the terpineol (8.75%), the limonene (6.35%), the isomenthone (5.75%) and the longifolene (5.47%) although Hadjiakhoondi et al., (2000),

have found like major component for the same leaves, the carvone (22.40%), the linalool (11.25%) and the limonene (10.80%) in the same country.

Table 2. Chemical composition of *Cymbopogon citratus* essential oil

Elements	KI	Percentage (%)
6-methyl-hep-5-en-2-one	985	1.2
Myrcene	**991**	**10.4**
Limonene	1031	**-**
β-ocimene	1036	0.4
6.7-epoxymyrcene	1091	0.2
Pirillene	1098	0.1
Linalol	1100	0.5
2.2-octa-3.4-dienal	1106	0.1
Vervenol	1140	0.1
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**
Geraniol	**1256**	**6.6**
Geranial	**1276**	**41.3**
neryl formate	1285	0.1
geranyl acetate	1378	2.4
Caryophyllene oxide	1587	0.1
Oxygenated monoterpens		**85.5**
Oxygenated Compounds aliphatic		**1.3**
Oxygenated Sesquiterpens		**0.2**
Total		**98.1**

Table 3. Chemical Composition of essential oil of *Mentha spicata*

Noun of the elements	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**
Piperitone	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
Non identified		**0.5**
Total		**99.86**

Chemical composition of the non volatile extracts

The phytochemical screening permitted to know the families of present compounds in the non volatile extracts of studied plants (table 4). Flavonoïdes, the polyphenols, the tannins (mainly the tannins catechic) and the volatile compounds have been identified in the two species. On the other hand, the alkaloids, the anthocyanes, the gallic tannins, the sterols and terpenes, the saponosides and the coumarines were identified in *Mentha spicata* extract but were absent in the one of *Cymbopogon citratus*. In the same way, anthraquinones and mucilages were identified in the leaves of *Cymbopogon citratus* and were present in trace the leaf of *Mentha spicata*. Let's signal that the cyanogenic derivatives were absent in the two plants species. Some similar results have been observed by Anjali and Sheetal (2013) that obtained the same compositions for the extract of *Mentha spicata*.

Table 4: Secondary metabolites compounds identified in non-volatiles extracts

Secondary Metabolites		*Cymbopogon citratus*	*Mentha spicata*
Alcaloïdes		-	+
Polyphenols		+	+
Flavonoïdes		+	+
Anthocyanes		-	+
Leuco-anthocyanes		+	-
Anthraquinones		+	±
Tannins	galliques	-	+
	catechiques	+	+
Sterols et terpenes		-	±
Mucilages		+	±
Saponosides		-	+
Coumarines		-	+
drifted cyanogenic		-	-
Volatile compounds		+	+

-absence; + presence

Microbiological characteristics of cheese

Analysis of table 5 revealed that all collected samples were contaminated by the coliforms that indicated fecal origin contamination. The presence of these germs showed the lack of the good practices of production and sale by some actors of the channel. The presence of *Staphylococcus spp* also confirms the preceding results, because these germs indicate cutaneous-mucous contamination and also inform on the level of applied hygiene during food product manipulation. Contamination by fungal was also noticed and was not only sanitary risks for the consumer because of their toxicity, but constitutes some important factors that cause merchant quality change of the product. The identification of these mildews permitted to show that they were from *Penicillium* species.

Table 5: Microbiological features of cheese

	Dantokpa	Godomey	Saint Michel	AFNOR Criteria
GAM	25.10^4	15.10^4	21.10^4	Nc
Yeasts	2.10^2	2.10^2	33.10^2	Nc
Mildews	2.10^2	10^2	10^2	Nc
Lactic Bacteria	41.10^2	16.10^2	12.10^2	Nc
Total coliformes	15	15	40	10^4
Fecal coliformes	18	17	14	Nc
E. coli	-	-	+	10
Staphylococcus	12.10^4	10.10^4	17.10^4	10^3

Nc: non considered

Antifungal and antibacterial activities of extracts

Results from evaluation of extracts antimicrobial activity at 100 mg/mL, 200mg/mL and 300mg/mL were presented in Figures 3-14 and table 6. It was necessary to notice that the increase of the inhibition rate explained the inhibition of the mycelial growth whereas its reduction translated the mycelial growth of tested mildew. Analysis of these figures and table showed that *C. citratus* essential oil of was fungicidal on the three tested fungal strains and bactericidal on *Escherichia coli* and *Candida albicans*. Non volatile extracts, would detain activities that the tested concentrations would not alloow to put in evidence. This strong activity antifungal and bactericidal of the chemical composition would result from their chemical composition characterized by a high concentration in monoterpenic aldehydes including the Neral (33%), Geranial (41. 3%) Geraniol (6.6%) for *C. citratus* and Limonene (16. 33%), Carvone (66.57%) for *M. spicata* and the non volatile extracts activities by the presence of the tannins, flavonoïdes and polyphenols in the two plants. Besides, these compounds are well known for their anti-infectious properties (Onawumi et al., (1984); Leichtnam, (1996); Schaneberg and Khan, 2002).

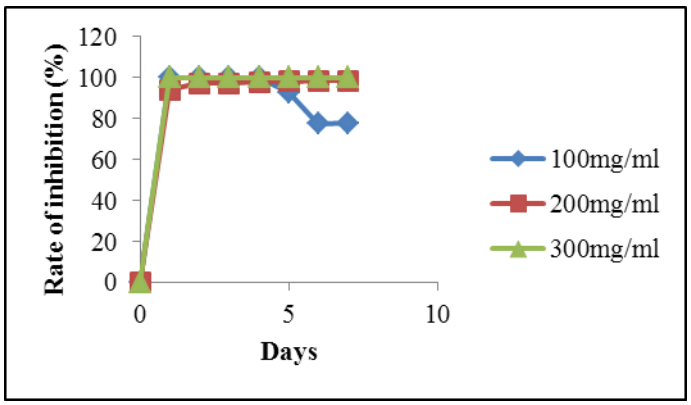

Figure 3. Inhibition rate of the mycelial growth of *Penicillium spp1* by *M. spicata* essential oil

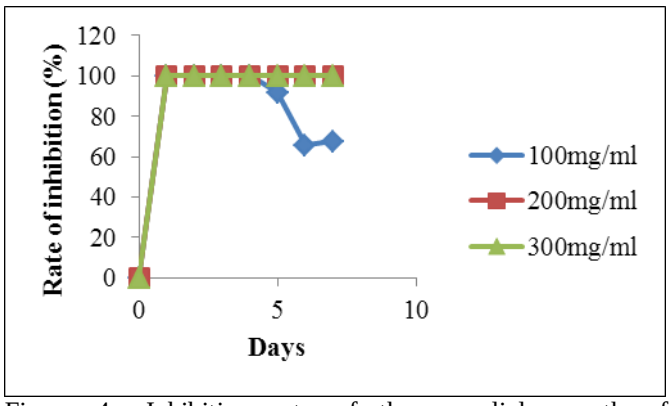

Figure 4 . Inhibition rate of the mycelial growth of *Penicillium spp2* by *M. spicata* essential oil

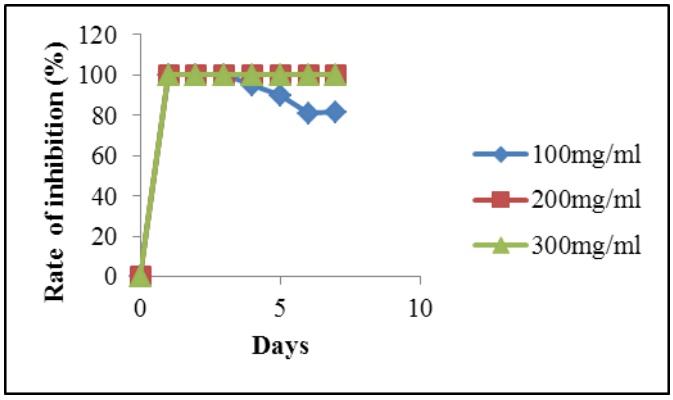

Figure 5. Inhibition rate of the mycelial growth of *Penicillium spp3* by *M. spicata* essential oil

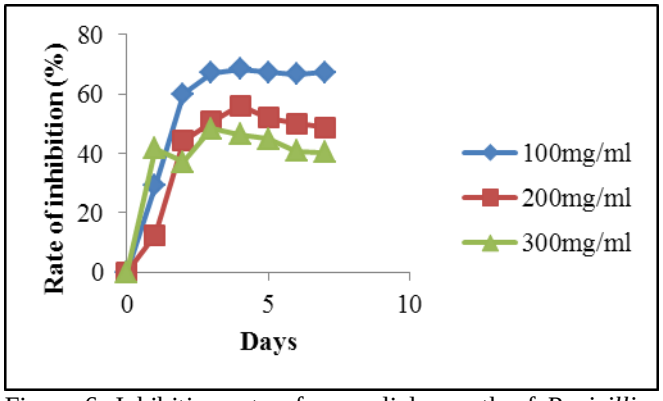

Figure 6. Inhibition rate of mycelial growth of *Penicillium spp1* by the non volatils extracts of *M. spicata*

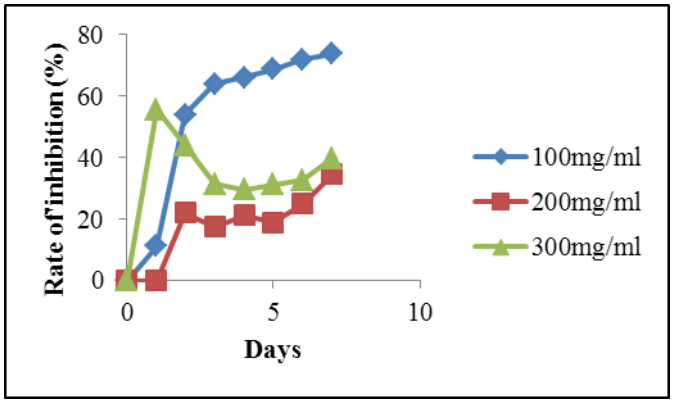

Figure 7: Inhibition rate of mycelial growth of *Penicillium spp2* by the non volatils extracts of *M. spicata*

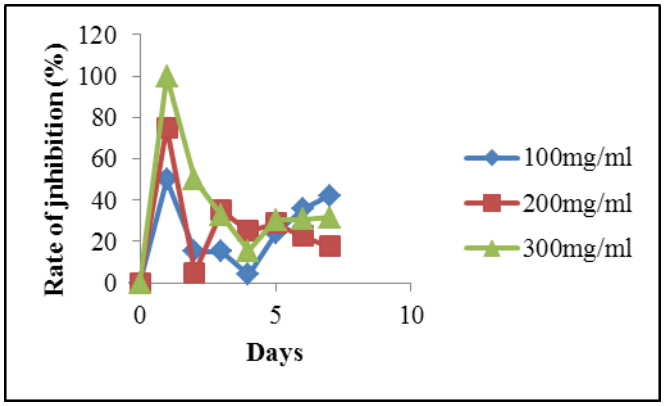

Figure 8 : Inhibition rate of mycelial growth of *Penicillium spp3* by the non volatils extracts of *M. spicata*

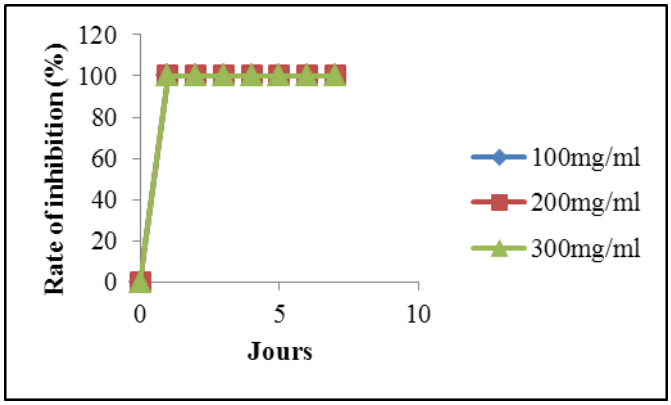

Figure 9 : Inhibition rate of the mycelial growth of *Penicillium spp1* by *C. citratus* essential oil

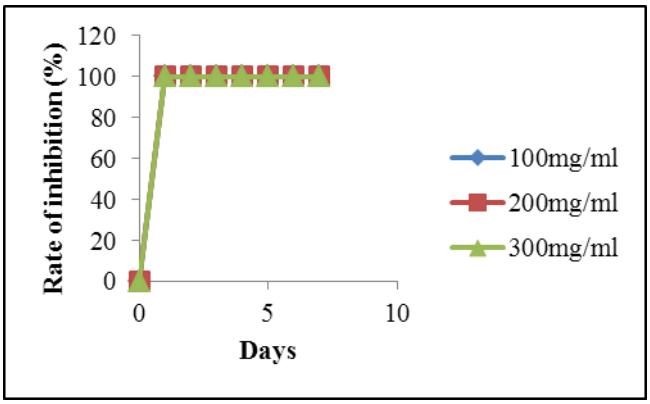

Figure 10: Inhibition rate of the mycelial growth of *Penicillium spp2* by *C. citratus* essential oil

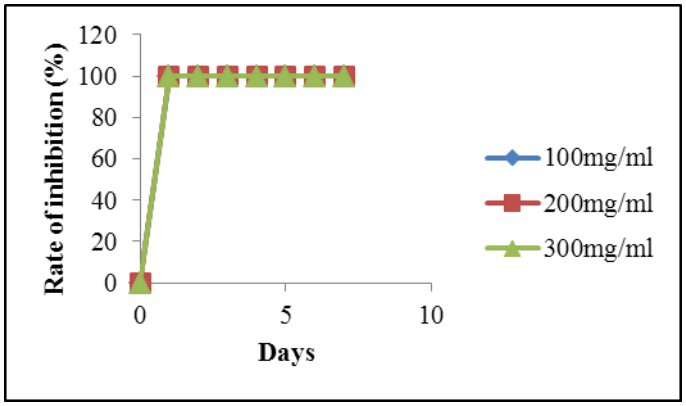

Figure 11. Inhibition rate of the mycelial growth of *Penicillium spp3*.by *C. citratus* essential oil

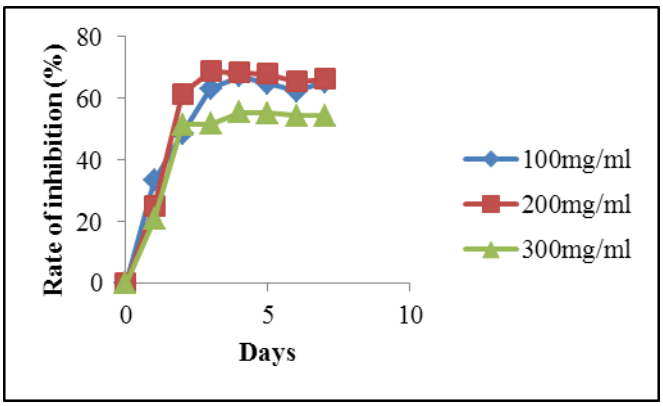

Figure 12. Inhibition rate of mycelial growth of *Penicillium spp1* by the non volatils extracts of *C. citratus*

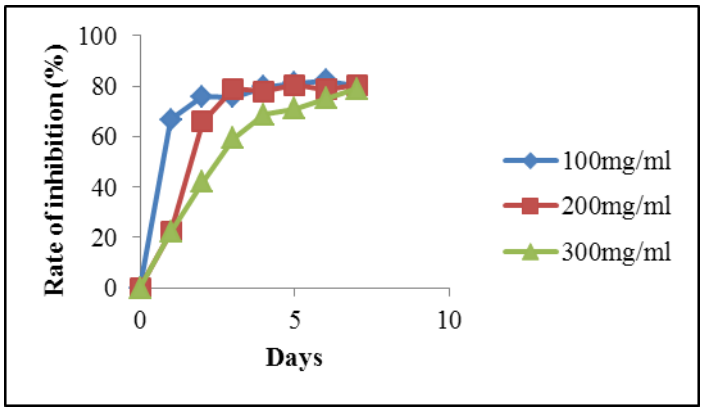

Figure 13. Inhibition rate of mycelial growth of *Penicillium spp2* by the non volatils extracts of *C. citratus*

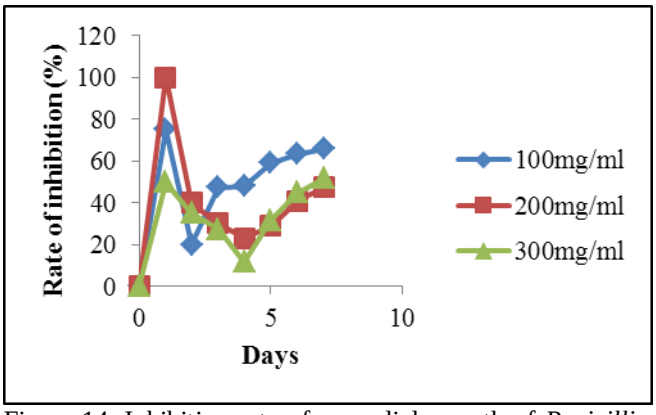

Figure 14. Inhibition rate of mycelial growth of *Penicillium spp3* by the non volatils extracts of *C. citratus*

Table 6: effect bactericid/bacteriostatic of the extracts on *C. albicans* and *E. coli*

		Candida albicans		*Escherichia coli*			
		MFC (mg/mL)	MfC (mg/mL)	MIC (mg/mL)	MBC (mg/mL)	CMB/CMI	Interpretation
Cymbopogon citratus	HE	100	-	100	100	1	Bactericidal
	NVE	>300	>300	>300	>300		-
Mentha spicata	HE	100	-	100	200	2	Bactericidal
	NVE	>300	>300	>300	>300		-

-: no activity; HE: Essential oil; NVE: Non Volatile Extract; MFC: Minimal Fungicidal Concentration; MfC: Minimal fongistatic Concentration; MIC: Minimal Inhibitory Concentration; MBC: Minimal Bactericidal Concentration.

Conclusion

This study showed the antimicrobial potential of the extracts from *Mentha spicata L.* and of *Cymbopogon citratus L.* on the pathogenic that cause adulteration of peuhl's cheese (waragashi). It is evident from it that the *Cymbopogon citratus L.* and *Mentha spicata L.* extracts had antifungal and antibacterial activity against varied strains isolated from cheese. However, the volatile extracts have shown pronounced antimicrobial activity on flora than non volatile extracts. Results also indicate that these extracts offer new perspectives in the conservation of cheese, without addition of compounds from chemical synthesis.

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