Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

J P P Journal of Pharmacognosy and Phytochemistry

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2017; 6(3): 693-697
Received: 18-03-2017
Accepted: 19-04-2017

Euloge S. Adjou
Laboratory of Research and Study in Applied Chemistry, Polytechnic School of Abomey-Calavi, University of Abomey-Calavi, 01 P.O.B: 2009, Cotonou, Benin

Dègnon René
Laboratory of Research and Study in Applied Chemistry, Polytechnic School of Abomey-Calavi, University of Abomey-Calavi, 01 P.O.B: 2009, Cotonou, Benin

Grace Mètomè
Laboratory of Research and Study in Applied Chemistry, Polytechnic School of Abomey-Calavi, University of Abomey-Calavi, 01 P.O.B: 2009, Cotonou, Benin

Edwige Dahouenon-Ahoussi
Laboratory of Research and Study in Applied Chemistry, Polytechnic School of Abomey-Calavi, University of Abomey-Calavi, 01 P.O.B: 2009, Cotonou, Benin

Correspondence
Euloge S. Adjou
Laboratory of Research and Study in Applied Chemistry, Polytechnic School of Abomey-Calavi, University of Abomey-Calavi, 01 P.O.B: 2009, Cotonou, Benin

Investigation on the efficacy of essential oil extract from leaves of *Lippia multiflora* on the quality of fresh cow milk during storage

Euloge S Adjou, Dègnon René, Grace Mètomè and Edwige Dahouenon-Ahoussi

Abstract
The aim of this study was to evaluate the effect of essential oil from fresh leaves of *Lippia multiflora* on the conservation of fresh cow milk during storage. The results of microbiological analyses revealed that coliforms and fungi were the most microorganisms identified from fresh cow milk in southern Benin. Chemical analysis by GC/MS of the components of the essential oil extracted from leaves of *Lippia multiflora* led to the identification of 19 components, characterized by Sabinene (14.4%), 1.8-cineol (50.6%) and α-terpinol (13.8%) as major components. Results obtained during the evaluation of the quality of stored cow milk by adding EO, indicated a significant decrease in the quantum of bacteria and fungi during storage, when compared to the control. However, the best antimicrobial potential were detected with essential oil concentration of 6.66µL.mL^{-1}. The essential oil of *Lippia multiflora,* with high antimicrobial property, offers a novel approach to the management of milk and derivate products during storage.

Keywords: *Lippia multiflora,* essential oil, fresh cow milk, conservation, Benin

Introduction
Among livestock products, cow's milk has a great socio-economic and nutritional importance. Secreted by mammals, milk is a complete food intended to provide energy, structural and immunological elements, during the early stages of life [1]. It is a dynamic system due to the presence of endogenous enzymes microorganisms and the existence of ionic equilibria which are dependent on certain conditions such as pH and temperature that conditioned the stability of the dispersed elements. These physical or biological changes lead to milk instability, which can be exploited during its transformation into dairy products such as fermented products, cheeses, creams and butters [1].
In Benin, milk and dairy products are very important in the diet and economy of agropastoral communities. They contribute more than 50% to the incomes of *Peulh* households [2]. However, milk is a very perishable food. Indeed, its pH, closed to neutrality, maked it very easily alterable by microorganisms and enzymes [3]. Its richness and fragility, made it an ideal environment where many microorganisms such as molds, yeasts and bacteria can reproduce very quickly. Its vitamins and fats contents can be altered under the influence of light, oxygen, or heating [3]. Milk is also contaminated by chemical pollutants such as heavy metals which modify its quality [4]. In the absence of an appropriate cold chain, the conservation of cow's milk during a long period becomes very difficult. This situation imposes on the actors of the milk's sector, the development of technics of conservation or transformation, more or less adapted to the socio-economic and environmental context [5]. Thus, new techniques for preserving fresh cow's milk are investigated [6]. These techniques include pasteurization or sterilization, which could have consequences on the nutritional and organoleptic quality of milk [5].
Several research have demonstrated the antifungal and antibacterial properties of essential oils [7, 8]. They are known both for their flavoring and antimicrobial properties, but also for their low toxicity compared to synthetic food additives [9]. Similarly, restrictions imposed by international organizations on the use of chemical synthesis preservatives due to health and environmental risks [10] increasingly lead to the use of essential oils in the conservation of food products [8].
Lippia multiflora is an aromatic plant, widespread in Africa. The characteristic aroma of its leaves, makes it consume as infusion or tea, hence its name of *Gambia tea* [11]. Several studies have reported the pharmacological, antimicrobial and antioxidant properties of this plant [12, 13].

Then, the present study aims to evaluate the efficacy of *Lippia multiflora* essential oil in preserving fresh cow milk in southern Benin.

Materials and methods

Collection of plant leaves

Plant materials used for essential oil (EO) extraction were fresh leaves from *Lippia multiflora*. Plants were collected at Savalou (center of Benin) and identified at the Benin national herbarium, where voucher specimens are deposited.

Essential oil extraction

The EO tested was extracted by the hydro-distillation method using Clevenger-type apparatus. The oil recovered was dried over anhydrous sodium sulfate and stored at 4 °C until it was used [14].

Gas chromatography–mass spectrometry analysis

The EO were analyzed by gas chromatograph (Perkin Elmer Auto XL GC; Waltham, MA, USA) equipped with a flame ionisation detector, and the GC conditions were EQUITY-5 column (60 m x 0.32 mm x 0.25 µm); H_2 as the carrier gas; column head pressure 10 psi; oven temperature program isotherm 2 min at 70 °C, 3 °C/min gradient 250 °C, isotherm 10 min; injection temperature, 250 °C; detector temperature 280 °C. Gas chromatography–mass spectrometry (GC-MS) analysis was performed using a Perkin Elmer Turbomass GC-MS. The GC column was EQUITY-5 (60 m x 0.32 mm x 0.25 µm); fused silica capillary column. The GC conditions were injection temperature, 250 °C; column temperature, isothermal at 70 °C for 2 min, then programmed to 250 °C at 37 °C/min and held at this temperature for 10 min; ion source temperature, 250 °C. Helium was the carrier gas. The effluent of the GC column was introduced directly into the source of MS and spectra obtained in the EI mode with 70 eV ionisation energy. The sector mass analyzer was set to scan from 40 to 500 amu for 22 s. The identification of individual compounds is based on their retention times, retention indices relative to $C_5 - C_{18}$ n-alkanes, and matching spectral peaks available in the published data [15].

Sampling of fresh cow milks

Fresh cow's milks were collected from two cattle farms located in Kpinnou and Athiémé (Southern Benin). Samples were aseptically collected in sterile glass bottles and transported to the laboratory in ice box at 5 °C and kept in the refrigerator at this temperature till analysis was carried out within 24 hour.

Microbiological analysis

For microbiological analysis, 25 g of sample and 225 ml of peptone water was added and homogenized. From the initial concentration, appropriate decimal dilutions were prepared and aliquots were plated in duplicates on various media. Plate count agar was used for the total bacterial count. Plates were incubated at 30 °C for 72 h. Desoxycholate was used for the total coliforms count and plates were incubated at 30 °C for 24 h. Desoxycholate was also used for the faecal coliforms count. In this case, plates were incubated at 44 °C and the identification was made using Eosine Methylene Blue (EMB) medium. Bair Parker medium was used for *Staphylococcus spp.* count and plates were incubated at 37 °C for 24h. Tryptone sulfite neomycin agar was used for anaerobic sulfito-reducer (ASR) count, and tubes were incubated at 37 °C for 24 h. Man Rogosa Sharpe agar (MRS) was used for *Lactobacillus spp* count, and plates were incubated at 30 °C for 72 h. Yeast and fungi count was performed using dilution plating method. Indeed, ten gram of each sample were added separately to 90 ml of sterile water containing, 0.1% peptone water. This was thoroughly mixed to obtain the 10^{-1} dilution. Further, 10fold serial dilutions up to 10^{-4} were made. 1 ml volume of each dilution was separately placed in Petri dishes, over which, 10 to 15 ml of potato dextrose agar amended with 60 µg/ml chloramphenicol (PDAC) was poured. The plates were incubated at 28 ± 2 °C for 7 days. After incubation, the number of colonies was tracked, using a colony counter. The number of microorganisms expressed as Colony Forming Units per milliliter (CFU/ml) was then determined by calculation, considering dilution factor. All media used for microbiological analysis were prepared as indicated by the manufacturer. After their isolation, bacteria were also controlled with API System (BioMérieux France)

Conservation of fresh cow milk with essential oil

To evaluate the conservation potentiality of the EO of *Lippia multiflora*, fresh cow milk collected at cattle sites of Kpinnou and Zinvié were mixed together to give a composite sample which was used for the tests. Three EO concentrations were tested. These are 3.33µl/ml, 5µl/ml and 6.66µl/ml $\mu L.mL^{-1}$. These concentrations were chosen taking into account the high fragrant nature of the EO and different results about its antimicrobial properties, reported in the literature [11, 13]. A negative control (fresh cow milk without EO) was also produced. Samples were placed at 25 °C, after homogenization. Periodical analyses were performed in other to evaluate the quality of milk during storage.

Statistical analysis

Experiments were performed in triplicate, and data analyzed are means ± SE subjected to one-way Anova. Means are separated by the Tukey's multiple range test when Anova was significant ($P<0.05$) (SPSS 10.0; Chicago, IL, USA).

Results and discussion

By hydrodistillation, fresh leaves of *Lippia multiflora* yielded 1.07 % of EO. Chemical analysis by GC and GC-MS analysis of EO enabled the identification of 19 components, (Table 1) representing 95.9 % of the EO. In the volatile extract, different groups of terpene and terpenoid, such as Monoterpenes hydrogenes (23.7%), oxygenated monoterpenes (65.3%), Sesquiterpenes hydrogenes (6.8%), oxygenated Sesquiterpenes (0.1%),) were detected. The EO has chemical composition characterized by Sabinene (14.4%), 1.8-cineol (50.6%) and α-terpinol (13.8%).

The result of microbial analysis revealed that the most contaminated microflora of fresh cow milk collected from two cattle's farming located at southern Benin are coliforms, yeast and fungi (Table 2). Indeed, total bacteria count are range from 3.301 to 3.602 log cfu/mL. *Lactobacillus spp* count are also ranged from 1.698 to 1.845 log cfu/mL. The level of contamination by coliforms detected in analyzed samples are about 3.00 log cfu/mL. Yeast and fungi contamination level are ranged from 1.477 to 1.698 log cfu/mL, with the absence of *Staphylococcus spp* and anaerobic sulfito-reducer bacteria in analyzed samples.

Results from the evolution of the total bacteria count, total coliforms count, *Lactobacillus spp*, yeasts and fungi in fresh cow milk conserved with the essential oil of *Lippia multiflora* during storage (Table 3 and 4) revealed that the antimicrobial activity depends on the dose of essential oil used. Indeed, with

the essential oil concentration of 6.66 µL.mL^{-1}, there is a considerable decrease in the microbial quantum of *Lactobacillus spp* during storage. Yeast and mold count have also considerably decrease in the milk during the preservation period with essential oil dose of 5 and 6.66 µL.mL^{-1}. These results indicated the effect of this essential oil in the inhibition of bacteria and fungi contaminating cow's milk in southern Benin.

The present study also revealed the proliferation of microorganisms of alteration in fresh cow's milk. Nowadays, the use of essential oils in the food industry is becoming increasingly important due to the many problems posed by chemical synthesis antimicrobials [8]. Then, this study explores the bioefficacy of EO of *Lippia multiflora* as the promising plant-based antimicrobial against fresh cow milk infecting fungal and bacteria growth. This EO was found to be effective against spoilage bacteria and fungi present in fresh cow milk in southern Benin. This bioefficacy may be due to the presence of some highly fungitoxic components in the oil such as terpenoids. Indeed, *Lippia multiflora* oil has a chemical composition characterized by terpenes and terpenoids as the main chemical groups. Several studies have indicated that terpenoids are a large group of antimicrobial compounds that are active against a broad spectrum of microorganisms [16]. Their antimicrobial activities are linked to their functional groups and it has also been reported that the hydroxyl group of phenolic terpenoids and the presence of delocalized electrons are important for the antimicrobial activity [16]. The high antimicrobial activity of this essential oil have also been reported by Bassolé *et al.* [9] in Burkina Faso and its used in the folk medicine to the treatment of diarrheas. Oussou *et al.* [17] also reported the antibiotic potential of this essential oil against enterobacteria such as *Escherichia coli, Shigella dysenteriae* and *Salmonella typhi*. In our study, GC–MS data depicted remarkable variation in the earlier reports on the oils [9, 12]. The chemical profile of EO is reported to be influenced by the harvest period, and by climatic, seasonal, and geographical conditions, which can significantly affect the amount and composition of the active constituents [8, 18]. Thus, the biologically active EOs should be qualitatively standardized before their recommendation for practical exploitation as has been done in the present investigation.

These results indicated that the industrial use of this essential oil could therefore be envisaged, with a view to stabilizing cow's milk in southern Benin. Essential oils are used nowadays in several agrifoodstuffs, in bakery and cheese [19] in sausage products [20] and in confectionery products [21]. The advantage of essential oils is their bioactivity in the vapor phase, a characteristic that makes them useful as possible smokes for the protection of stored products. Similarly, industrialized societies appear to be experiencing a green consumer trend, with fewer synthetic additives, due to the many nuisances associated with the use of antimicrobials from chemical synthesis [7]. Thus, spices and vegetable extracts as well as essential oils are known, since antiquity, as having many potentials such as antibacterial, antifungal, antioxidant, and are now increasingly used in food preservation [22] because of their relatively safe status and their wide acceptance by consumers [23].

Table 1: Chemical composition of *Lippia multiflora* essential oil investigated

Components	Kovats Index (KI)	Percentage (%)
α-thujène	920	0,3
α-pinène	929	3,7
sabinène	969	14,4
β-pinène	973	1,9
myrcène	981	2,9
1,8-cinéol	1033	50,6
γ-terpinène	1040	0,3
terpinolène	1084	0,2
linalol	1092	0,3
camphèn-6-ol	1123	0,1
pinocarvone	1162	0,2
terpinen-4-ol	1175	0,3
α-terpinol	1192	13,8
α-cubebène	1378	0,1
aromadendrène	1450	4,2
germacrene - D	1487	2,1
isoledene	1519	0,2
δ-cadinene	1524	0,2
(E) - nerolidol	1556	0,1
Total		95,9

Table 2: Microbiological quality of collected fresh milk

Parameters	Values (log cfu/mL)		OMS Criteria
	Zinvié	Kpinnou	
Total bacteria count	3.602	3.301	<5
Lactobacillus spp	1.845	1.698	<5
Total coliforms count	3	3	<2
Faecal coliforms count	00	00	<2
Staphylococcus spp	00	00	<2.477
ASR bacteria count	00	00	Absence
Mold and fungi count	1.698	1.477	-

Table 3: Effect of the essential oil of *Lippia multiflora* on the Total Bacteria count and Total Coliforms count of fresh cow milk during storage

Days	Microbiological parameters (log cfu/ml)							
	Total Bacteria count				Total coliforms count			
	0	3.33µl/ml	5 µl/ml	6.66 µl/ml	0	3.33µl/ml	5 µl/ml	6.66 µl/ml
1	3.477	3.477	3.477	3.477	3	3	3	3
3	3.698	3.378	3.176	2.176	3.698	2	1.901	1.701
6	4.041	3.371	3.277	2.177	3.778	1.510	1.477	1.603
9	4.477	3.277	3.079	2.079	4.477	1.420	1.113	1.501
12	4.477	3.177	3.077	1.467	4.477	1.315	1.112	1
15	4.477	2.711	2	0	4.477	1.107	0	0

Table 4. Effect of the essential oil of *Lippia multiflora* on the *Lactobacillus spp.* Count and yeast and fungi count of fresh cow milk during storage

Days	Microbiological parameters (log cfu/ml)							
	Lactobacillus spp				yeast and fungi			
	0	3.33µl/ml	5 µl/ml	6.66 µl/ml	0	3.33µl/ml	5 µl/ml	6.66 µl/ml
1	1.778	1.778	1.778	1.778	1.602	1.602	1.602	1.602
3	3.903	3.698	1	0	2.93	1.313	1.343	1
6	5.477	3.301	0.8	0	3	1.313	0	0
9	4.477	3.202	0.6	0	3	1	0	0
12	4.477	0.313	0.6	0	3	0.9	0	0
15	5.054	0	0	0	3	0.4	0	0

Conclusion

This work underlined the bioactivity of EO of fresh leaves of *Lippia multiflora* from Benin as a bacteria and fungal growth suppressor in fresh cow milk. Different major components such Sabinene (14.4%), 1.8-cineol (50.6%) and α-terpinol (13.8%) were present in the volatile extract. Based on its antimicrobial potential, this natural plant product may successfully replace synthetic chemicals and provide an alternative method to protect fresh cow milk as well as other milk derivate products against the contamination and the growth of bacteria and fungi.

Acknowledgments

The authors are grateful to the Department of Food Engineering of Polytechnic School of Abomey-Calavi University for the financial support.

References

1. Jeannet R, Croguennec T, Schuck P, Brulé G. *Sciences des aliments : Biochimie – Microbiologie – Procédés – Produits,* 2008, 456.
2. Sessou P, Alitonou G, Farougou S, Azokpota P, Kaneho S, Youssao I *et al*. Bioefficacy of *Cymbopogon citratus* essential oil against foodborne pathogens in culture medium and in traditional cheese wagashi produced in Benin. International Research Journal of Microbiology. 2012; 3(12):406-415.
3. Dossou J, Hounzangbe-Adote S, Soule H. Production et transformation du lait frais en fromage peuhl au Bénin. Guide de bonnes pratiques. Bénin - GRET - CAD/FSA, 2006, 33.
4. Dahouenon-Ahoussi E, Degnon GR, Adjou ES. Investigation on the Impact of Environmental Pollution on the Contamination of Cow's Milks From two Cattle Farming Areas of Southern Benin. J Anim Sci Adv. 2015; 5(7):1357-1362.
5. Kèkè M, Yèhouénou B, de Souza C, Sohounhloué DCK. Evaluation of hygienic and nutritional quality of peulh cheese treated by *Sorghum vulgaris* (L) and *Pimenta racemosa* (Miller) extracts. Scientific Study & Research, 2009; 10(1):29-46.
6. Abdenouri N, Idlimam A, Kouhila M. Etude hygroscopique du lait en poudre. Revue des Energies Renouvelables SMSTS 2008; 08:35-44.
7. Burt S. Essential oils: their antibacterial properties andpotential applications in foods-a review, Int. J Food Microbiol. 2004; 94:223–253.
8. Soumanou MM, Adjou ES. Sweet Fennel (*Ocimum gratissimum*) Oils. In: Preedy, V.R. (Ed.), Essential Oils in Food Preservation, Flavor and Safety. Academic Press, 2016, 765-773.
9. Bassolé IHN, Lamien-Meda A, Bayala B, Tirogo S, Franz C, Novak J *et al*. Composition and antimicrobial activities of *Lippia multiflora* Moldenke, *Mentha x piperita* L. and *Ocimum Basilicum* L. essential oils and their major monoterpene alcohols alone and in combination. Molecules 2010; 15:7825-7839.
10. Yèhouénou B, Noudogbessi J-P, Sessou P, Avlessi F, Sohounhloué D. Etude chimique et activités antimicrobiennes d'extraits volatils des feuilles et fruits de *Xylopia aethiopica* (Dunal) A. Rich. Contre les pathogènes des denrées alimentaires. Journal de la Société Ouest-Africaine de Chimie. 2010; 029:19-27.
11. Yehouenou B. Composition chimique et activités antimicrobiennes d'extraits végétaux du Benin contre les micro-organismes pathogènes et altérants des denrées alimentaires. Thèse de Doctorat, Faculté des sciences et Techniques, Université d'Abomey-calavi, Benin, 2012, 221.
12. Owolabi MS, Ogundajo A, Lajide L, Oladimeji MO, Setzer WN, Palazzo MC. Chemical Composition and Antibaterial Activity of the Essential Oil of *Lippia Multiflora* Moldenke from Nigeria. Rec. Nat. Prod. 2009; 3(4):170-177.
13. Bonou J, Baba-Moussa F, Adéoti Z, Ahouandjinou H, Dougnon V, Dossa D *et al*. Antimicrobial activity of essential oils of *Lippia multiflora*, *Eugenia caryophyllata*, *Mentha piperita* and *Zingiber officinale* on five oral-dental microorganisms 2016; 5(5):271-276.
14. de Billerbeck VG, Roques CG, Bessière JM, Fonvieille JL, Dargent R. Effect of Cymbopogon nardus (L) W. Watson essential oil on the growth and morphogenesis of Aspergillus niger. Can. J. Microbiol. 2001; 47:9–17
15. Adams RP Identification of essential oil components by gas chromatography ⁄ mass spectrometry. Allured, Carol Stream, 2007, 22.
16. Dorman HJD, Deans SG. Antimicrobial agents from plants: antibacterial activity of plant volatile oils. J Appl. Microbiol. 2000; 88:308–316
17. Oussou KR, Yolou S, Boti JB, Guessennd KN, Kanko C, Ahibo C *et al*. Etude Chimique et Activité Antidiarrheique des Heuiles Essentielles de deux Plantes Aromatiques de la Pharmacopée Ivoirienne. European J of Scientific Res. 2008; 24(1):94-103.
18. Kpadonou-Kpoviessi BGH, Yayi-Ladekan E, Kpoviessi DS, Gbaguidi F, Yehouenou B *et al*. Chemical variation of essential oil constituents of *Ocimum gratissimum* L. from Benin, and impact on antimicrobial properties and toxicity against *Artemia salina* Leach. Chem. Biodivers 2012; 9:139-150.
19. Vazquez BI, Fente C, Franco CM, Vazquez MJ, Cepeda A. Inhibitory effects of eugenol and thymol on *Penicillium citrinum* strains in culture media and cheese. Int. J Food Microbiol. 2001; 67:157-163.
20. Quintavalla S, Vicini L. Antimicrobial food packaging in meat industry. *Meat Science* 2002; 62:373-380.

21. Lanciotti R, Gianotti A, Patrignani F, Belleti N, Guerzoni ME, Gardini F. Use of natural aroma compounds to improve shelf life and safety of minimally processed fruits. Trends in Food Science and Technology 2004; 15:201-208.
22. Bounatirou S, Smiti S, Miguel MG, Faleiro L, Rejeb MN, Neffati M *et al*. Chemical composition, antioxidant and antimicrobial activities of the essential oils isolated from Tunisian *Thymus capitus* Hoff. et Link. Food Chemistry 2007; 105:146-155.
23. Sawamura M. Aroma and functional properties of Japanese yuzu (Citrus junos Tanaka) essential oil. Aroma Research 2000; 1(1):14-19.