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Antimicrobial activity of three essential oils from Benin on five oral germs: Test of mouthbaths

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ABSTRACT

In Beninese pharmacopeia, many plants are considered as having some antimicrobial properties. The first objective of this study is, to evaluate the antimicrobial activity of three essential oils on five bucco-dental germs and to carry out mouthwash with essential oils. The second objective is to determine their Inhibiting Minimal Concentrations (IMC), Bactericidal Minimal Concentrations (BMC) and chemical compositions. The results of this work revealed that the Inhibiting Minimal Concentrations (IMC's) of the oils vary between 0,078 and 2,50 mg/Ml. The mouth wash carried out at 2°/₀₀ essential oil was efficient on the five germs with dilutions between ½ and 1/16. The chemical analysis of the most active essential oil shows the presence of: estragole (84,98%), geranial (34,92%), neral (30,75%), para-cymene (21,89%), 1,8-cineole (10,09%) et thymol (8,46%).The most active essential oils can be used in aromatherapy to improve health and the well-being.

Key words: essential oils, Inhibiting Minimal Concentration, Bactericidal Minimal Concentration, mouthwash.

INTRODUCTION

Tooth decay and periodontal diseases have always been considered as the two main complaints in oral health [1]. Their distribution and severity vary both countrywide or regionally [2]. In many developing countries, the access to the oral health care is limited. African and Asian countries must tackle several very serious bucco–dental complaints, such as noma, acute necrotizing ulcerative gingivitis, and pre-cancerous and cancerous lesions of the buccal cavity, immediately [3]. Furthermore, Africa and Asia have the highest prevalence of HIV/AIDS, which's oral health manifestations are much spilled and very frequent [4]. The oral illnesses represent a major public and sociological health problem. A varied range of antibiotics and antiseptics are used to fight these oral germs.

The classical treatment of these illnesses is extremely costly, being at the fourth rank in terms of Health costs in most industrialized countries. Only the costs of paediatric treatment of these diseases would surpass the total health care budget in many countries to weak income [5]. This brings the population to resort to traditional medicine, namely to plants. Nowadays, more than 80% of the African populations seek help form herbal medicine for their health issues [6]. Plant extracts, containing essential oils, represent a natural marvel with their medicinal properties [7].

The present study investigated antimicrobial activities of essential oils of the three most commonly used plants to traditionally treat oral pathology in Benin and on the formulation of mouth baths having antiseptic activity. In order to do so, the essential oils of these three plants have been extracted and chemically analysed. Their in vitro bacteriostatic and bactericidal activities have been tested on five oral germs. Finally, mouth baths from efficient essential oil have been formulated and bacteriostatic and bactericidal activities have also been tested for the five oral germs.

MATERIAL AND METHODS

Plant

The plant material contains essential oils in the following parts: leaves, fruits, flowers or rhizomes of different plant species. These are:

- *Cymbopogon citratus* (DC.) Stapf (Poaceae) ; *Ocimum basilicum* L. (Lamiaceae) ;
- *Lippia multiflora* Moldenke (Verbenaceae)

Identification of the plants has been made by the National herbarium of the University Abomey-Calavi. The plant harvest has mainly been done in Porto Novo, Sèmè, (ouémé department), Kétou (Plateau department).

Microorganisms

The antibacterial and antifungal activities have been evaluated on reference ATCC (American Type Culture Collection) and IP (Institute Pasteur of Paris) strains. The latter's are:

- gram positive cocci
- *Micrococcus luteus* ATCC 10240, *Staphylococcus aureus* ATCC 29213
- Bacillus negative gram
- *Proteus mirabilis* ATCC 24974, *Pseudomonas aeruginosa* ATCC 27853
- Mushroom (yeast)*Candida albicans* IP 4872

Essential oil extraction

The essential oils have been extracted by hydrodistillation with a Clavenger-type apparatus during 180 and 240 min according to the part of the plants. The extracted essential oils have then been dried on anhydrous magnesium sulphate and been stored at 4° C in colourful small bottles. The yield of oil for every extraction has been calculated.

Chromatographic essential oil analysis

The method used is gas chromatography analysis coupled to mass spectrometry. The analysis has been done in an automatic way with a gas chromatograph (DELSI IGC 121°C) equipped with a capillary column CP WAX 52 CBS (25 m of length and 0,3 mm of interior diameter) and of a flame ionization detector (FID). The vector gas is nitrogen with 1 mL/min flow in the exit of the column, the report of leak is 1/60 = 1mL/min and the pressure is 0, 8 bar. The combustible gas is hydrogen (flow, 30 mL/min and pressure, 1 bar). The fuel gas is the compressed air; its flow is 300 mL/min under a pressure of 1 bar. The injection is at 240° C and the detector 250° C. The isotherm landing is at 50° C during 5 min with a pressure gradient of 2° C/min during 85 min until a final temperature of 220°C. The volume of injected oil is 0, 05 µLS.

This chromatograph is coupled to a mass spectrometer. It is a mass detector HEWLETT PACKARD (HP) type 5970 with ionization by electronic impact (70 eV), equipped with a non-polar capillary column (DB-1) in melted silica of 25 - m long and 0,23 mm of interior diameter. The vector gas is helium with a flow of 0,9 mL/min. The isotherm landing is then of 1 min in 60° C the pressure gradient is 3°C/min until 180° C. The volume of injected oil is of 1 µL.

In vitro survey of the antimicrobial and antifungal activity

The sensitivity of *Micrococcus luteus*, *Staphylococcus aureus*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Candida albicans* to the different essential oils has been tested by the micro dilution method. The Minimal

Inhibitory Concentration (MIC), which is the smallest concentration, at which there is no visible growth to the naked eye, has been determined.

Essential oil emulsion preparation
An initial solution of 20 µL/mL (20 mg/mL) of essential oil was prepared in nourishing broth or Sabouraud. We added the tween 80 there at the rate of 5%, all was homogenized on vortex.

Preparation of suspended microbial solution
After isolation, a colony of germs was dissolved in 10 mL sterile distilled water in a test tube. We then have made a set of dilution with sterile distilled water. The obtained dilution was plated on nourishing agar or Sabouraud. The cultures have been incubation for 24 h at 37° C for the bacteria and for 48 h at ambient temperature for *Candida albicans*. The number of microorganisms expressed as Colony Forming Units per millilitre (cfu/ml) was counted. The dilution that gave a concentration of 10^6 germs/mL for the bacteria and 10^7 germs for *Candida albicans* was kept.

Preparation of the microplates and determination of the MIC and MBC
Micro dilutions on 24 well plates allowing visual appreciation of the microorganism's growth have been used. We distributed 950 µL of nourishing broth in all wells and added 50 µL of the initial solution of essential oil (20 mg/mL) in each of the wells of the first column and 950 µL of nourishing broth in each of the wells of the third column at the rate of oil by row. Then, dilutions - successive homogenizations from the third column of well by well, arranged by row, in order to have a dilution in geometric series of reason 2 has been done. Finally, all wells, with the exception of those of the first column, were sowed with 50 µL of an inoculum at 10^6 germs/mL

Two control wells have been used. The microplate was covered and has been incubated at 37° C during 24 hours for the bacteria and the read-out has been made by comparison between controls well and tests well. For *Candida albicans*, the culture was made on Sabouraud broth at 27° C during 48 hours. The Minimal Inhibitory Concentration (MIC) has been determined. The Minimal Bactericidal Concentration (MBC) was also determined. . In the case of visible growth, we compared the multiplication of the germs to those of the control agar. The MBC is the smallest concentration for which there was at least 0,01% of germs survivors.

Survey of the mouth bath
Formulation of the mouth bath
The studied mouthbath has been realized as previously described [8].

Table I

For 100 ml of solution we used:
Essential Oil ……. 0.2 ml
Citric acid ……..0.6 g
Aspartam (Canderel®)…… 0,4 g
- Sodium Hydroxide ……0.6 g
- Methyl Salicylate ……...0.15 g
- Tween 80……0.5 g
- Alcohol 95°……5 ml
- Water completed to…… 100 ml

Determination of MIC and MBC
The used methodology is identical to the one used for the essential oils. From the solution of mouth bath, we realized a series of dilutions. A 1/128 dilution, of two mouth baths sold in the pharmacy: Eludril® and Givalex®, were also tested on our germs.

RESULTS

Essential oil extraction
The table II below presents the yielded extractions of different plant species. It is evident from this table that the most elevated yield was achieved with the essential oil of *Lippia multiflora* (2.32%), followed by *Cymbopogon citratus* (0.7%) and the weakest one (0.5%) was gotten from *Ocimum basilicum*

Table II: Yields of the essential oil extractions

Names	Family	Yields (%)
Cymbopogon citratus	Poaceae	0.7
Lippia multiflora	Verbenaceae	2.32
Ocimum basilicum	Lamiaceae	0.5

Essential oil survey

Determination of the Minimal Inhibitory Concentration (MIC)

According to the results related to the determination of the minimal inhibitory concentrations of our essential oils (Table III), we can deduct that the essential oils of *Cymbopogon citratus* and *Ocimum basilicum* have the weakest MIC (0.078 to 0.156 mg/mL). They are followed by those of *Lippia multiflora* (0.078 to 2.50 mg/mL).

The most sensitive germ is *Micrococcus luteus* (MIC= 0.312 mg/mL) and the most resistant is *Pseudomonas aeruginosa*.

Table III: Results of the determination of the MIC of the different essential oils.

Essential oils	MIC (mg/mL)				
	Staphylococcus Aureus	*Proteus mirabilis*	*Micrococcus luteus*	*Pseudomonas aeruginosa*	*Candida albicans*
Cymbopogon citratus	0.156	0.078	0.078	0.078	0.078
Ocimum basilicum	0.078	0.078	0.078	0.156	0.156
Lippia multiflora	0.078	0.312	0.156	2.50	0.156

Determination of the Bactericidal Minimal Concentration (MBC)

The results of the MBC determination are consigned in table IV. Globally, the MBC are superior or equal to MIC. The essential oils of *Cymbopogon citratus* and *Ocimum basilicum* are the most bactericidal (0.078 to 0.625 mg/mL). They are followed by those of *Lippia multiflora* (0.078 to 2.50 mg/mL) .

In conclusion, the most inhibitoriests essential oils are at the same time the most bactericidal ones. The most sensitive germ always remains *Micrococcus luteus* (0.078 to 0.625 mg/mL) and most resistant remains *Pseudomonas aeruginosa* (0.156 to 2.50 mg/mL).

Table IV Results of MBC determination of the different essential oils.

Essential oils	MBC (mg/mL)				
	Staphylococcus Aureus	*Proteus mirabilis*	*Micrococcus luteus*	*Pseudomonas aeruginosa*	*Candida albicans*
Cymbopogon citratus	0.156	0.625	0.078	0.312	0.078
Ocimum basilicum	0.312	0.312	0.078	0.156	0.156
Lippia multiflora	0.078	0.312	0.625	2.50	0.156

Survey of mouth bath

The mouth baths have been prepared with the three essential oils. These are: *Cymbopogon citratus*, *Ocimum basilicum*, *Lippia multiflora.*

Determination of the minimal inhibitory concentration of the mouth baths

The results of the determination of the MIC of the mouth baths are presented according to the dilutions that permitted the determination of the MIC.

According to the table V, we can say that the mouth baths based on *Cymbopogon citratus* and *Ocimum basilicum* are the most efficient ones (dilutions 1/2 to 1/16 correspondent to the concentration 0.001 µg/mL in 0.000125 µg/mL). They are followed by the *Lippia multiflora* mouth bath (0.001µg/mL in 0.00025 µg/mL).

Finally, the Givalex® and Eludril® mouth baths taken as references inhibited the germs at a dilution of 1/128.

Table V: Determination of the Minimal Inhibitory Concentration of the mouth baths

Mouth baths	MIC				
	Proteus mirabilis	*Micrococcus luteus*	*Staphylococcus aureus*	*Pseudomonas aeruginosa*	*Candida albicans*
Negative control	1/4	1/16	1/16	1/4	IS
Cymbopogon citratus	1/8	1/16	1/8	1/8	1/2
Lippia multiflora	1/4	1/8	1/8	1/4	1/2
Ocimum basilicum	1/8	1/16	1/8	1/4	1/4

IS: Initial Solution

Determination of the minimal bactericidal concentration of the mouth baths

The results of the MBC of the mouth baths are presented in the table VI. The mouth bath based on *Cymbopogon citratus* is the most efficient (0.001 in 0.00025 µg/mL). It is followed the ones containing *Ocimum basilicum* (0.001 in 0.00025 µg/mL) and *Lippia multiflora* (0.001 in 0.0005 µg/mL).

Table VI: Determination of the minimal bactericidal concentration of the mouth baths

Mouth baths	?BC				
	Proteus mirabilis	*Micrococcus luteus*	*Staphylococcus aureus*	*Pseudomonas aeruginosa*	*Candida albicans*
Negative control	1/4	1/8	1/16	1/4	IS
Cymbopogon citratus	1/8	1/4	1/8	1/8	1/2
Lippia multiflora	1/4	1/2	1/2	1/4	1/2
Ocimum basilicum	1/8	1/4	1/8	1/4	1/2

IS: Initial Solution

Essential oil analysis

The results of the chromatographic analysis are summarized in the tables VII-VIII.

Essential oil of *Cymbopogon citrates*:

The analysis of the table VIII shows that the major composites are neral (30.75%) and geranial (39.42%) among the 82.81% of oxygenated compounds. The identified compounds represent 94.30%.

Table VIII: Chemical composition of *Cymbopogon citratus* essential oil

N°	Retention time (min)	Kovats Indices	Identified components	Percentage
1	11.914	**986**	6-methyl-hept-5-en-2-one*	1.71%
2	**12.086**	**991**	**Myrcene**	**10.78%**
3	13.651	**1037**	(Z)-beta-ocimene	0.37%
4	14.000	**1048**	(E)-beta-ocimene	0.24%
5	15.453	**1092**	6.7-epoxymyrcene*	0.17%
6	15.741	**1100**	linalol*	0.81%
7	16.953	**1140**	trans-4-caranone*	0.11%
8	17.273	**1151**	p-menth-3-en-9-ol*	0.14%
9	17.355	**1154**	citronellal*	0.30%
10	19.487	**1226**	nerol*	0.11%
11	19.563	**1229**	citronellol*	0.33%
12	**19.946**	**1242**	**neral***	**30.75%**
13	20.227	**1252**	geraniol*	5.58%
14	**20.785**	**1272**	**geranial***	**39.42%**
15	23.615	**1378**	geranial acetate*	2.87%
16	24.790	**1424**	beta caryophyllene	0.23%
17	25.065	**1435**	trans alpha bergamotene	0.17%
18	26.423	**1490**	(Z.E)-alpha-farnesene	0.10%
19	28.703	**1587**	oxide de caryophyllene*	0.12%
			Identified components	**94.30%**
			Non Identified components	**5.70%**
			***Oxygenated components**	**82.41%**

Essential oil of *Lippia multiflora*

The oxygenated compounds mentioned in the table XII represent 53.62% of the compounds identified (98,98%). Para cymene (21, 89%), 1,8-cineole (10,09%), thymol (8.46%) and geranial (7,17%) are the major constituents.

Table XII: Chemical composition of *Lippia multiflora essential* oil

N°	Retention time (min)	Kovats Indices	Identified components	Percentage
1	9.851	**927**	alpha thujene	1.10%
2	10.112	**935**	alpha pinene	0.98%
3	11.510	**974**	sabinene	2.38%
4	11.679	**979**	beta pinene	0.36%
5	11.768	**982**	oct-1-en-3-ol*	0.35%
6	11.920	**986**	6-methyl-hept-5-en-2-one*	1.83%
7	12.079	**990**	myrcene	1.34%
8	13.030	**1018**	alpha terpinene	0.63%
9	**13.320**	**1027**	**para cymene**	**21.89%**
10	13.454	**1031**	limonene	0.99%
11	**13.572**	**1035**	**1.8-cineole (eucalyptol)***	**10.09%**
12	14.010	**1048**	(E)-beta-ocimene	0.33%
13	14.415	**1060**	gamma terpinene	4.70%
14	14.823	**1073**	cis sabinene hydrate*	0.15%
15	15.752	**1101**	linalol*	1.97%
16	17.361	**1154**	citronellal*	0.18%
17	18.260	**1183**	terpinene-4-ol*	0.60%
18	18.696	**1198**	alpha terpineol + myrtenol*	2.02%
19	19.485	**1226**	citronellol*	0.45%
20	19.541	**1228**	nerol*	0.71%
21	19.894	**1240**	neral*	4.86%
22	20.214	**1252**	geraniol*	3.80%
23	20.724	**1270**	**geranial***	**7.17%**
24	21.356	**1292**	**thymol***	**8.46%**
25	21.574	**1300**	carvacrol*	1.26%
26	22.803	**1347**	Thymyl acetate*	6.07%
27	22.877	**1350**	eugenol*	0.29%
28	23.295	**1366**	carvacryle acetate *	0.22%
29	23.618	**1378**	alpha copaene	0.62%
30	23.653	**1380**	geranyle acetate *	0.47%
31	23.875	**1388**	beta bourbonene	0.24%
32	23.985	**1392**	beta elemene	0.24%
33	24.800	**1425**	beta caryophyllene	2.64%
34	25.515	**1454**	beta-(E)-farnesene	2.63%
35	25.691	**1461**	allo aromadendrene	0.50%
36	25.797	**1465**	9-epi-(E)-carophyllene	0.35%
37	26.132	**1479**	gamma muurolene	0.18%
38	26.308	**1486**	germacrene-D	1.43%
39	26.697	**1502**	alpha muurolene	0.31%
40	26.891	**1510**	beta bisabolene	0.61%
41	27.159	**1521**	delta cadinene	0.94%
42	27.874	**1552**	elemol*	0.39%
43	28.714	**1588**	caryophyllene oxide *	1.26%
44	29.348	**1616**	humulene II epoxide *	0.22%
45	29.453	**1620**	torilenol*	0.34%
46	30.331	**1660**	beta eudesmol*	0.28%
47	30.427	**1665**	alpha cadinol*	0.19%
			Identified components	**98.98%**
			Non Identified components	**1.02%**
			***Oxygenated components**	**53.62%**

Essential oil of *Ocimum basilicum*

Estragole (84.98%) is the major constituent of the identified compounds in the table XIV. The oxygenated compounds represent 93.12% of the identified compounds (99.18%).

Table XIV: Chemical composition of *Ocimum basilicum* essential oil

N°	Retention time (min)	Kovats Indice	Identified components	Percentage
1	10.116	**935**	alpha pinene	0.17%
2	11.683	**979**	beta pinene	0.16%
3	12.082	**990**	myrcene	0.33%
4	13.449	**1031**	limonene	0.37%
5	13.562	**1035**	1.8-cineole*	1.52%
6	14.013	**1048**	(E)-beta-ocimene	0.25%
7	15.752	**1101**	linalol*	0.44%
8	16.402	**1122**	exo fenchol*	0.35%
9	17.272	**1151**	camphre*	1.02%
10	**18.862**	**1204**	**methyl chavicol (estragole)***	**84.98%**
11	19.333	**1220**	exo-fenchyle acetate *	0.78%
12	20.391	**1258**	para anisaldehyde*	0.18%
13	21.204	**1287**	acetate de bornyle*	0.61%
14	23.993	**1392**	beta elemene	0.26%
15	24.198	**1400**	methyl eugenol*	0.37%
16	25.083	**1436**	trans alpha bergamotene	3.71%
17	26.348	**1487**	tran-beta-bergamotene	0.28%
18	27.066	**1517**	gamma cadinene	0.54%
19	28.339	**1572**	para-methoxy-cinnamaldehyde*	1.57%
20	28.583	**1582**	spathulenol*	0.19%
21	28.719	**1588**	caryophyllene oxide *	0.11%
22	29.451	**1620**	1.10-di-epi-cubenol*	0.16%
23	30.034	**1647**	epi-alpha-cadinol*	0.84%
			Identified components	**99.18%**
			Non Identified components	**0.82%**
			***Oxygenated components**	**93.12%**

DISCUSSION

From the results of our study, it results that the majority of investigated microorganisms is sensitive to the essential oils. The MIC varies from 0,078 to 10mg/mL. The most active is the *Cymbopogon citratus* essential oil, followed by *Ocimum basilicum* and *Lippia multiflora*. The most sensitive microorganism is *Micrococcus luteus* and most resistant is *Pseudomonas aeruginosa.*

In general, the Gram negative bacteria proved to be more resistant to the tested products than the Gram positive bacteria. This sensitivity was more marked for the Gram positive bacteria to the essential oil compounds have already been observed by several authors [9,10].

The essential oil of *Ocimum basilicum* has a MIC of 1, 25 mg/mL against our germs. These results are different of those of KPAVODE [11] that showed that these oils don't have an effect on the germs notably *Staphylococcus aureus* and *Candida albicans*. They are also different from the essential oil of *Cymbopogon citratus* that gave MIC of 0,156 mg/mL against 5 mg/mL on the same germs. These differences could be explained by the fact that the tested essential oils don't have the same chemical composition.

To be able to compare the biological activities of our essential oils, we indicated for each oil its major components. The essential oils don't have the same chemical composition. Some are rich in 1,8-cineole: This is for example the case for *Lippia multiflora* (10,09%), while the one of *Cymbopogon citratus* is rich in neral and geranial (30,75% and 39,42% respectively).

The major constituent from the chromatographic analysis of the essential oil of *Cymbopogon citratus* are: myrcene

10,78%, the neral 30,75%, and the geranial 39,42% are compliant to NFT 75-231: 1974 standard.

DONGMO *et al* in 2001 [12], had attributed the efficiency of the citrus extracted essential oils to their high content of neral and geranial. The essential oil of *Cymbopogon citratus* studied in our work also presents a strong content in neral and geranial and is part of the most active essential oils. Thus, our work confirms those of our predecessors.

The essential oil of *Lippia multiflora* is rich in 1,8-cineole and showed a MIC lower than 1mg/mL. These results were different to those of LENS-LISBOA et al [13] who in 1987 showed that the 1,8-cineole and the para - cymene have a MIC superior to 1mg/mL on *Staphyloccocus aureus*.

The essential oil of *Clausena anisata*, rich in estragole (96,09%), was revealed to be efficient on the germs, contrary to the results of KPAVODE [11] who didn't find any activity in spite of a content of 83,19% in estragole. In the same way, they were also different for *Ocimum basilicum* rich in estragole (84,98%) against estragole (31,33%), linalol (24,00%) and eugenol (21,67%) content. The difference in chemical compounds could explain the differences between the antimicrobial activities. To conclude, the mouth baths prepared confirmed the essential oil activity. The mouthbaths based on chlorhexidine (Eludril®) and hexetidine (Givalex®) have a bactericidal activity on the germs studied in spite of the strong dilution. These results could be explained by the fact that the essential oils are not pure substances contrary to hexetidine and to chlorhexidine and that it would be necessary to search the active molecules of these essential oils.

CONCLUSION

The antimicrobial properties of three essential oils were investigated on five oral germs: *Micrococcus luteus*, *Staphylococcus aureus*, *Proteus mirabilis*, *Pseudomonas aeruginosa* and *Candida albicans.* The obtained results showed that the majority of our essential oils was active on the microorganisms of our survey with MIC and MBC that vary from 0,078 to 2,50mg/mL. The most active essential oils were those of *Cymbopogon citratus* and of *Ocimum basilicum*. The sensitivity of the bacteria varies from a strain to another. The most sensitive strain is *Micrococcus luteus* (MIC = CMB = 0,078 mg/mL) with the essential oil of *Cymbopogon citratus* and of *Ocimum basilicum*, most resistant is *Pseudomonas aeruginosa* (MIC = CMB= 2,50 mg/mL) for the *Lippia multiflora* oil.

The research of the chemical composition of the essential oils showed the presence of terpenes (terpinene, myrcene, limonene), aldehydes compounds (citronellal, neral, geranial), phenolics (estragole, thymol), alcohols (geraniol, linalol) and cetonics (menthone). The most active essential oils were those of *Cymbopogon citratus*, *Lippia multiflora* and *Ocimum basilicum* respectively rich in neral, paracymene, and estragole.

The mouth baths prepared with 2‰ essential oil were efficient on the five germs to included dilutions between 1/2 et1/16 and permit to consider an improvement of the local phytotherapy in the fight against the oral diseases.

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