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BIOACTIVITY OF ESSENTIAL OILS FROM MEDICINAL PLANTS OF CAMEROON AND THEIR COMBINATION AGAINST INFANT DIARRHEA INDUCED BY BACTERIA

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Abstract

Background: In Cameroon, one of the most common childhood illnesses after malaria is diarrhea, which generally has a bacterial origin. In rural areas, plant utilization is often one of the first ways of treatment though, with no scientific bases. Therefore, the aim of this work was to evaluate the antibacterial activities of nine essential oils (EOs) of Cameroonian plants and their combinations against some strains responsible for diarrhea.
Material and Methods: To determine the bacterial species generally involved in childhood diarrheal infections, a retrospective study was done. The EOs of *Eucalyptus globulus*, *Cymbopogon citratus*, *Xylopia aethiopica*, *Thymus vulgaris*, *Ocimum canum*, *Cananga odorata*, *Citrus medica*, *Citrus paradisi* and *Citrus reticulata* were screened against the most incriminated bacterial species using the spot-on-agar test and microdilution methods. Some of the EOs with good antibacterial activity were analyzed by Gas Chromatography and Gas Chromatography/Mass Spectrometry. According to their composition, *Thymus vulgaris* and *Eucalyptus globulus* EOs were combined with that of *Cymbopogon citratus* using the ratios 2/1; 1/1; 1/2 (w/w) and tested against on bacterial growth.
Results: The retrospective study showed that 10.8% of infant diarrhea was caused by *Escherichia coli*, *Salmonella typhimurium* and *Salmonella paratyphimurium*. The Minimum inhibition Concentrations (MIC) of single EOs was between 0.78 and 25 mg/mL which were observed to be greater than those of their combinations which ranged between 0.195 and 6.52 mg/mL. The active essential oils contained mainly hydrocarbonated and oxygenated monoterpens.
Conclusion: The good antibacterial effects of two *Thymus* combinations Th_1 and Th_2 observed on *Salmonella typhimurium* and *Salmonella paratyphimurium* suggest their used in aromatherapy to cure bacterial diarrhea.

Key words: Infant diarrhea, Essential oils, Combinations, Antibacterial activity.

Abbreviations: EO: Essential oils; GC/MS: Gas chromatography-mass spectrometry; DMSO: Dimehtylsulfoxide; GC: Gas chromatography; LRI: Linear retention indices on a HP-5 column. HNC: Cameroon National Herbarium; SRF: Société des reserves forestières; Cam: Cameroon.

Introduction

Essential oils (EOs) are aromatic volatile substances of plants, known for their antibacterial activities. They are recognized as non-toxic in aromatherapy and safe by the Food and Drug Administration (FDA) (Franchomme, *et al*., 2011; Kaloustian and Hadji-minaglou, 2012). Many "*in vitro"* and "*in vivo"* studies report a high efficacy of these substances against bacterial infection or food spoilage hence their use in aromatherapy (as alternative or complementary medicine) and food industries (Nelson and Mildenhall, 1967; Nyegue, 2006; Uhart *et al.*, 2006; Kouamé *et al*., 2008; Prabhu *et al.*, 2009). Due to increase in infant mortality resulting from bacterial induced diarrhea and the recurrent problems of bacterial resistance, the use of EOs could be a good alternative to antibiotics and food preservatives (Wembonyama, 1997; Dupeyron, 1997; OMS, 2010; Fabre, 2010). In Cameroon particularly in Yaoundé city, 36.9 % of children less than five years suffer from bacterial diarrhea and in this developing country, lifestyle and foodstuffs are a real challenge for the population (Dennehy, 2005; IFMT, 2005; Nguendo, 2009). Therefore looking for alternative means of preventing and treating diarrheal diseases is an opportunity to give value to natural medicine by testing these plant products against some bacterial strain.

However, to obtain an efficient effect like that of antibiotics, some EOs required a higher concentration "*in vitro"* or *"in vivo"* reasons why their combinations may often help to optimize their activities (Essia, 2008; Gutierrez *et al.,* 2008). Even though many studies have already shown the efficiency of EOs as antibiotic substances, there are few reports about the scientific proofs of their use in combinations in aromatherapy. It has been explained that essential oils combination has the advantage to have the effect at many sites of action on bacterial cells with several mechanisms of action. It therefore result an increase of the effect and a limitation of the phenomenon of resistance (Bassolé *et al*., 2010; Bassolé *et al.,* 2011)**.**

Xylopia aethiopica Duval *(Annonaceae)*, *Cananga odorata* Hook and Thomson *(Annonaceae), Thymus vulgaris* L. *(Lamiaceae), Ocimum canum* Sims (*Lamiaceae*) *Eucalyptus globulus* Labill (*Myrtaceae)*, *Cymbopogon citratus* DC Stapf (*Poaceae*), *Citrus medica* L. (*Rutaceae*), *Citrus paradisi* Macf (*Rutaceae*) and *Citrus reticulate* Blanco (*Rutaceae*) are nine plants belonging to five genus, usually use as natural medicine (in Cameroon), cosmetics and spices. *Xylopia aethiopica* is a tropical tree which can reach 15 to 45 m of height and 60 to 75 cm of size. The tree produces 4 to 9 seeds which are wrapped into fruits. It is largely distributed in tropical zones especially Central Africa where it is used as spice during cooking or as drug in pharmacopoeia for their therapeutic virtues to treat many diseases (Tchiégang and Mbougueng, 2005). *Thymus vulgaris* is a very aromatic plant reaching 20 to 50 cm of height (Tchoumbougnang *et al.,* 2009). In Cameroon, this plant is cultivated in home gardens and found around the home (Tchoumbougnang *et al.,* 2009). In addition to its culinary virtues as aromatic herb, thyme has many other properties; for example its infusion is used against infections of the respiratory system (cough, bronchitis, asthma). *Ocimum canum* (Exotic basilic) is a tropical plant giving a strong aromatic odor. It is very fragrant and cultivated near village's gardens (Tchoumbougnang *et al.,* 2009). Its leaves and floral luminary parts are used to prepare pectoral tonic infusions while the leave extract or chewing is used to treat colds and headaches. *Cananga odorata* is a tree about three meters in height that produces flowers ranging in color from green to yellow and green fruits with a strong odor. In Cameroon, people plant it in gardens, parks and near homes because of its fruity and sweet flower fragrance which emerges at dawn or early in the morning. Its essential oil is largely used in cosmetic industries to produce products of high added value. *Eucalyptus* is a beautiful tree native to Australia and have been implant in Europe mainly Spain; it is a tree of about 30 to 35 meters in height, with a straight and smooth trunk (Warot, 2006). In Cameroon, *Eucalyptus globulus* is cultivated in the Western Regions where its leaves are used to treat infections of the respiratory tract and cough. *Cymbopogon citratus* (lemon grass) is a plant with thin and linear leaves coloured green releasing a fresh and relaxing smell. It is a traditional cooking ingredient in Cameroon and its leaf extracts are widely used as relaxing herbal tea which helps to fight against stress, relieve stomach aches. In Central Africa, *Cymbopogon citratus* is mostly cultivated around houses because the flavor of its leaves is toxic for mosquitoes (Tchoumbougnang *et al.*, 2009). Species of the genus *Citrus* are trees or shrubs less than 4 m to 12 m high. The fruit is wrapped with peel which contains a lot of EOs. In Cameroon fruits of the genus *Citrus* are used as medicines to fight against cough and as natural source of vitamins. The aim of this study is to evaluate the antibacterial activities of the essential oils of these nine plants and their combinations against some bacterial strains responsible for infant diarrhea.

Material and Methods

Plant material

The plant samples used for this study were bought in Yaoundé market. *Xylopia aethiopica*, *Thymus vulgaris*, *Ocimum canum* and *Citrus medica*, were harvested between the 11th and 22nd of October 2012 and identified at the Cameroon National Herbarium under the code numbers: 59700/HNCI, 25746/SRF/Cam, 15866/SRF/Cam and 65106/HNC respectively. *Citrus paradisi* and *Citrus reticulata* which are not local plants of Cameroon were harvest at the same period and identified in IRAD (Institute of Research Agriculture and Development). *Eucalyptus globulus, Cymbopogon citratus* and *Cananga odorata* were harvested between 09 and 30 September 2012 and their identification numbers were 4077/SRFK, 48536/SFR/Cam and 42250/HNC at the Cameroon National Herbarium.

Microbial strains

The antimicrobial activity of the essential oils was individually tested against three Gram negative bacterial strains including *Escherichia coli ATCC* 25922, *Salmonella typhimurium* and *Salmonella paratyphimurium.* The uncoded strains were clinically isolated and identified at *Centre Pasteur du Cameroun* and Yaoundé Central Hospital.

Etiological survey

A five weeks retrospective study from July 25th to August 25th 2012 at the Yaounde University Teaching Hospital Centre permitted the evaluation of the number of stool culture realized for children between the ages of 0 to 5 years. The patients were reviewed retropectively with the approval of (CHU) University Hospital of Yaoundé registered under code 416 AR/DG/DGA/DMT. The retrospective review was focused between March 2009 and July 2012.

Data collection and definition

The work consisted of compiling the results of bacterial analysis of stool cultures and to note the enteropathogenic bacteria isolated from each sample. Data and results from reported patients were gotten from the recording registry of bacteriological laboratory. After noting the ages, diarrheal stool were described that is; wet, bloodly or glairy character followed by bacterial analysis. The number of diarrhea cases were counted and then, the number of those with pathogenic bacteria. The results from stool cultures were used to identify the strains implicated in the diarrheal diseases.

Patient's sample characteristics

Patient's age was one of the criteria taken into account to include or not the patient in the study. The macroscopic descriptions of the patient's stool (wet stools) done by the doctor were used to distinguish cases of diarrhea. Each child under 5 years was included in our study when the macroscopic description of the stool was made and the result of bacterial stool analysis provided. A child was classified as having suffered from diarrhea when the description of stools revealed the presence of mucus, blood or water (Case Example: loose stools or molded; mucoid and liquid diarrhea). The result of the bacterial analysis showed different bacterial diarrhea cases and characterization by Apie gallery of sample were used to confirm the presence of pathogenic bacteria in case of diarrhea.

Essential oil extraction

The collected plants were subjected to steam distillation using a Clevenger type apparatus with the plant sample completely immersed into water and heated to boiling. The essential oil evaporated together with water vapor and then separated by decantation on cooling. Finally, the essential oil was dried over anhydrous sodium sulfate, filtered and stored in dark in an amber glass vial until the time of use. After the extraction, yields were calculated.

Determination of the chemical composition of essential oils by Gas Chromatography (GC) and Gas Chromatography coupled to Mass Spectrometry (GC-MS)

The GC were carried out on a Variant CP 3380 gas chromatograph equipped with a flame ionization detector (FID) adjusted at 250^{o}C coupled to two types of apolar columns (silica capillary): polar HP-5 J and W (Agilent (5%-phenyl-95% methyl polysiloxane) of capillary column 30m x 0.25mm thickness and film thickness of 0.25µm) and Supelcowax 10 (polyethylene glycol, Supelco Inc, Bellfonte, PA) fused capillary (internal diameter 30m x 0.25mm, 0.25µm film thickness). Nitrogen was the carrier gas used at a constant flow rate of 0.8 mL/min with injector regulated in split mode at 220^{o}C. The exit ratio was 1:100 (0.1µL of pure EO). The injector temperature was 220^{o}C while that of the detector was 250^{o}C. The temperature was then programmed at 50^{o}C to 200^{o}C at a ramp of 5^{o}C/ min and then maintained at 200^{o}C for 10 minutes: The entire set-up was coordinated by a computer system with the COPPASS software that ensured its functioning and follow-up of the chromatographic analyses from which quantitative data were obtained from FID area percent data as described by Agnaniet *et al.*, 2011.

The GC-MS was performed on a gaseous phase chromatograph using a Hewlett-Packard (GC 5890 series II) equipped with a HP-5(5% phenyl-95%methyl polysiloxane) fused capillary silica column (internal diameter of 30m x 0.25mm, film thickness of 0.25µm) interfaced with another fused silica capillary DB-Wax (internal diameter of 30m x 0.25mm, 0.25µm film thickness). The mass detector was of the quadrupole Model 5972 and the following conditions were used: ionization energy was 70eV, column temperature programmed from 50^{o}C to 200^{o}C, ramp of 5^{o}C/ min and first maintained at 50^{o}C for 2 minutes. The injection and MS transfer line temperatures were fixed at 220^{o}C and 180^{o}C respectively. Helium was used as the carrier gas at a flow rate maintained at 0.6 mL/ min; inlet: split, 1:10 (1 µL of a 10:100 CH2Cl2 solution), ionization voltage of 70eV; electron multiplier 1460eV, mass scan range 35-300 a.m.u, scan rate 2.96 scan/s. Injection of 0.1µL of pure EO. The percentage composition of the EO was computed by the normalization method from the GC-FID peak areas, assuming an identical mass response factor for all compounds. A series of n-alkanes were used as reference points. The identification of the EO components was based on comparison of their relative retention index with published data in the literature and by matching their mass spectra with these published data (Adams, 2012).

Antibacterial assays

Firstly, a screening (qualitative activity) of the active EOs was done by using the spot-on-agar test (NCLSI, 2012). EO extracts were diluted in Tween 20 at 5% (v/v) to obtain a concentration of 100 mg/mL. Then 10 µL was spotted under sterilized paper disks and deposited on agar surface inoculated with the different bacterial strains cited above. The spotted plates were incubated at 37° C for 24h after which the inhibited zones were measured and the mean of the triplicate was express in mm.

Secondly, three active EOs were used in combination. The essential oils of *Thymus vulgaris, Eucalyptus globulus* were each combined with *Cymbopogon citratus* as the common base of combination. Two groups of 3 combinations namely Th_1, Th_2 and Th_3 made by *Thymus*/*Cymbopogon citratus* and Eu_1, Eu_2 and Eu_3 made by

Eucalyptus/*Cymbopogon citratus* as shown in Table 1 were done using the proportions of 2/1; 1/1; 1/2 (w/w) and retested in a solid medium as the individual EOs at a concentration of 100 mg/mL against the same bacterial strains.

Table 1: Combinations and EOs proportions

EOs used	***Cymbopogon/ Eucalyptus***			***Cymbopogon/ Thymus***		
Combinations	Eu_1	Eu_2	Eu_3	Th_1	Th_2	Th_3
Proportions (w/w)	(2/1)	(1/1)	(1/2)	(2/1)	(1/1)	(1/2)

Statistical analysis

Results are expressed as Means ± SD. The comparisons between treated samples were assessed by one-way ANOVA. Statistically significant differences were considered for P values <0.05.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MIB)

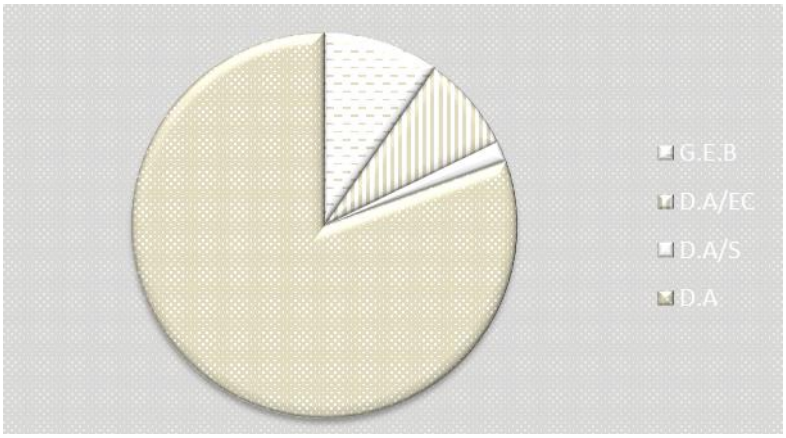
The microdilution method (quantitative activity) was used for the determination of MIC. The MIC was defined as the lowest concentration of essential oil inhibiting visible bacterial growth after 18 or 20 h incubation at 37°C (CLSI, 2012). Into each well, 100 µL of broth (L: S-BIOTECH® CA9212 USA) enriched with 5% red phenol was added. Then, 100 µL of each active essential oil or combination was added in every first well of the microplate. Geometric dilutions ranging from 50 to 0.781 mg/mL were carried out and subsequently, 100µL of media containing 10^6 UFC/mL of the indicator strain was added to all wells to yield 25 to 0.0152 mg/mL of concentration. The plates were then incubated at 37°C for 24 h. For each active EO or combination, the experiment was done in triplicate. A color change from red to yellow was indicative of bacterial growth. To obtain the MBC, 20 µL of each well colored red was spotted on agar surface of plates and incubated at 37°C for 24 h. The MBC was defined as the lowest concentration of essential oil where 10 or less than 10 colonies growing in the plate were counted.
The ratio MBC/MIC was calculated and according to the values obtained in inhibition tests, the essential oil was classified as bactericidal: when MBC/MIC was 1 or 2; bacteriostatic MBC/MIC was 4 and finally tolerant when the values are in the range $16 \leq MBC/MIC \geq$ (Fauchère and Avril, 2002).

Results and Discussion

Etiological survey

During the retrospective study, we were given 127 infant stool cultures analyzed between March 2009 and July 2012. Figure 1 showed bacterial repartition of infant diarrhea in Yaoundé's C.H.U. From these, the macroscopic descriptions showed that 111 were diarrheal cases in which 12 (10.8%) were induced by bacteria and 89.2% by unknown causes. The bacterial strains that have been isolated from the infant's stools were *Escherichia coli* (9.0%) and *Salmonella* spp (1.8%) as mentioned in Figure 1.

The analysis of results showed that in the sub-Saharan area, *E. coli* and *Salmonella* spp are still the main causes of infant diarrhea. This result is in accordance with that of Luki *et al.* which also showed that 12.0% of bacteria are responsible for infant diarrhea in Kinshasa city where *Salmonella* spp (61.5%) and *E. coli* (19.0%) are the most incriminated (Luki *et al.*, 1986). The presence of *Escherichia coli* and *Salmonella* spp in stool samples can be related to unhygienic conditions of air or dirty food and water intake in Yaoundé city; because these two strains can easily live in water and foods. This can be justified by the bacteriological profile of water consumed in Yaoundé and its neighbourhoods in which *E. coli* (5.15%) and *Salmonella* spp (1.30%) were isolated (Nguendo, 2010). According to the clinical statistics, 10.8% of infant diarrhoea was caused by bacteria; this is comparable to the 10.4 % obtained by Sanou *et al*., in the Ouagadougou city (Sanou *et al.*, 1999). In the same way, Diouf *et al*. reported that 10.5% of infant diarrhea was caused by enteropathogenic bacteria (Diouf *et al*., 1990). In this way, bacterial diarrhea constitutes a public health challenge in sub-Sahara area where there is a real problem of contaminated water. Although the percentage of bacterial strains isolated from infant's stool samples was only 10.8%, it is probable that other strains have not been taken into consideration during the bacterial analysis of stool samples. Gram positive bacteria also known to be bacteria of food spoilage, cannot cross the enteric tract because of the biliary salts. This could justify their absence in stool samples collected even if it were during or after the diarrhea symptoms.



D.C= diarrhoeal cases**; B.D** = bacterial diarrhoea. **B.D/E** = bacterial diarrhoeaby *E. coli*; **D.A/S** = bacterial diarrhoeaby *Salmonella ssp.*

Figure 1: Bacterial repartition infant diarrhea in Yaoundé's C.H.U between Mach 2009 and June 2012

Essential oil extraction

The extraction yields ranged between 0.24 for *Citrus reticulata* and 3.09 % for *Xylopia aethiopica.* The table 2 shows that the leaves of *Eucalyptus globulus* 0.81%) are richer in EOs than the *Lamiaceae* (*Thymus vulgaris* 0.32% and *Ocimum canum* 0.34%). Some plant organs contain more essential oil than others; like *Xylopia aethiopica* seeds which got the highest quantity of essential oils 3.09%.

These results showed that coming from different organs, the proportion of EOs present in a plant can vary not only through the botanical family but also from one species to another (Bruneton, 1999; Mohammedi, 2006). It can be confirmed with Hellal's works which showed that the dry pericarps of some *Citrus* species have more EOs than others (Hellal, 2011).

Table 2: The oil yield extracted from different part of plant samples collected

Plants samples	Part of plant used	Date of harvest	Yield (W/W) %
E. globulus	Dry leaves	27-Sept-12	0.81
C. paradisi	Pericarps	10- Oct-12	0.36
C. medica	Pericarps	07- Oct-12	0.64
C. citratus	Aerial part	09-Sept-12	0.28
C. reticulata	Pericarps	12- Oct -12	0.24
X. aethiopica	Dry seeds	11- Oct-12	3.09
C. odorata	Fresh flower	30-Sept-12	0.35
T. vulgaris	Airial part	13- Oc -12	0.32
O. canum	Fresh leaves	13- Oct -12	0.34

Chemical analysis of essentials oils

The results of the chromatographic profile of the main bioactive class of chemical components of essentials oils are shown in table 3. Generally, the chemical composition of these EOs showed that they are constitute mainly hydrocarbonated and oxygenated monoterpenes except *Cananga odorata* and *Eucalyptus globulus* which in addition to monoterpenes contain sesquiterpenes.

The GC profiles of the EO showed that the main constituents were α-pinene (20.0%), globulol (7.6%), caryophyllene oxide (16.2%), α-sesquiphellandrene (11.1%), camphor (10.3%), eucalyptol (10.2%) for *Eucalyptus globulus* ; limonene (47.6%), β-pinene (18.2%), γ-terpinene (8.1%), terpinen-4-ol (4.3%), linalol (3.1%) for *Citrus medica* ; geranial (49.2%), neral (34.3%), myrcene (5.9%), geraniol (1.9%) for *Cymbopogon citratus*; germacrene D (1.5), farnesyl acetate (14.1), germacrene B (4.9), linalool (17.4), geranyl acetate (9.2) for *Cananga odorata* andthymol 45.5%, linalol 4.2%, *p*-cymene 25.0%, γ-terpinene 5.3% for *Thymus vulgaris.*

The *Cananga odorata* EO was dominated by oxygenated monoterpenes (26.6%), hydrocarboned and oxygenated sesquiterpenes (20.5%). The same profile is common to a species from Madagascar which contained farnesyl acetate, germacrene D and linalool as major components (AFNOR, 2005). The chemical composition of *Citrus medica* EO revealed the presence of hydrocarboned monoterpenes represented 73.9 %, limonene (45.6 %), β-pinene (18.3 %), δ-terpinene (8.1), oxygenated monoterpenes 7.3 %, terpinen-4-ol (4.3 %) and linalool (3.1 %). This result obtained is common to *Citrus* species which have as main component limonene (Moufida and Marzouk, 2003). Geranial (49.2 %), neral (34.3 %) and myrcene (5.9 %) were the major constituents of almost *Cymbopogon citratus* EOs. The findings on the composition of EOs from the leaves of *Cymbopogon citratus* were similar to those previously

reported except those of Tchoumbougang *et al.* which was rich in geraniol (15.6 %), geranial (39.3 %), neral (21.9 %) and myrcene (14.0 %) (Tchoumbougnang, *et al.,* 2009). Other reports indicated that neral and geranial are the main characteristic constituents of *Cymbopogon citratus* (Menut, 2000; Koba *et al*., 2004). In fact, during the period of plant growth the composition of its secondary metabolites changes in response to its needs or environmental factors (Bruneton, 1999). The EO of *Eucalyptus globulus* contains less eucalyptol or 1,8-cineole (10.3%) than that found in literature. This weak proportion of oxygenated sesquiterpene like globulol and caryophyllene oxide known as toxic at high concentration for children can be more appreciated in aromatherapy to treat infant diseases (Warot, 2006). The EO of *Thymus vulgaris* was mainly composed of thymol (45.5%). This means that the thymol chemotype is different from *p*-cymene chemotypes or carvacol chemotype. This difference in composition can be observed during the different growth periods of *Thymus* species. Some reports indicate that at the end of growth, *Thymus vulgaris* contains more *p*-cymene than thymol and at the beginning more thymol than *p*-cymene (Vampa *et al.*, 1988). The GC profile of *Xylopia aethiopica* EO showed as main class of components hydrocarboned monoterpenes 63.9% (sabinene 23.7%, β-pinene 17.2%, α-pinene 9.4% and limonene 13.7%) and oxygened monoterpenes 12.0% (Eucalyptol).The same composition is mentioned by Bakarnga-Via *et al.* showing that the EO of *Xylopia aethiopica* contains between 72.4% and 64.8% monoterpenes with major components being sabinene (4.8-14.5%) and limonene (21.0%), β-pinene (24.6.9-28.2%) and terpinen-4-ol (10.0-15.1%) (Bakarnga-Via *et al.*, 2014).

Table 3: The main components and chemical classes of active EOs

Plant s amples	Main components	(%)	Chemical classes of compounds	(%)
E. globulus	α-Pinene	20.0	Hydrocarboned Monoterpenes	20.0
	Globulol	7.6	Oxygenated Sesquiterpenes	23.8
	Caryophyllene oxide	16.2		
	α-Sesquiphellandrene	11.1	Hydrocarboned sesquiterpene	11.1
	Camphor	10.3	Oxygenated Monoterpenes	20.5
	Eucalyptol	10.2		
C. medica	Limonene	47.6		73.9
	β-Pinene	18.2	HydrocarbonedMonoterpenes	
	γ-Terpinene	8.1		
	Terpinen-4-ol	4.3	Oxygenated Monoterpenes	7.4
	Linalol	3.1		
C. citratus	Geranial	49.2	Monoterpenic aldehydes	83.5
	Neral	34.3		
	Myrcene	5.9	Hydrocarboned Monoterpene	5.9
	Geraniol	1.9	Monoterpenic alcohol	1.9
X. aethyopica	Sabinene	23.7	Hydrocarboned monoterpenes	64.1
	β-Pinene	17.2		
	α-Pinene	9.4		
	Limonene	13.7		
	Eucalyptol	12.0	Oxygenated Monoterpene	12.0
C. odorata	Germacrene D	1.5	Hydrocarboned Sesquiterpene	6.4
	Germacrene B	4.9		
	Linalol	17.4	Oxygenated Monoterpenes	26.6
	Geranyl acetate	9.2		
	Farnesyl acetate	14.1	Oxygenated sesquiterpene	14.1
T. vulgaris	Thymol	45.5	Oxygenated Monoterpenes	49.7
	Linalol	4.2		
	p-Cymene	25.0	Hydrocarbonated Monoterpenes	30.3
	γ-Terpinene	5.3		

Antibacterial assays
Sensitivity tests

The antimicrobial activities of the essential oil against a set of seven strains were assessed qualitatively by the presence or absence of inhibition zones. From the sensibility tests, six EOs and 4 combinations were active against all the strains.

These activities could be explained by the presence of the some compounds known for their antibacterial properties. In fact, alcohols such as linalool, aldehydes such as citral, hydrocarboned monoterpenes and phenols present in EO samples are the groups of compounds which have generally an antibacterial activity (Dorman and Deans, 2000; Rhayour *et al*., 2003). These secondary metabolites act by inducing the cell lysis (phenols), denaturing proteins and lipids of cell membrane (alcohols), aldehydes stop protein activity and DNA replication while hydrocarboned monoterpenes disrupt membrane potential (Dorman and Deans, 2000; Da Silva, 2010). All these classes or groups of

compounds have been identified during the characterization of our EO samples and hence justify their antibacterial activity against the sensitive strains.

The results in table 4 show that there is significant difference between essential oil activities in general. Particularly, the *Eucalyptus globulus, Thymus vulgaris* and *Cymbopogon citratus* oils exhibited a better activity on tested strains than the other EOs with ($P<0.05$). The more sensitive strains were *Salmonella typhimurium* (diameters of inhibition between 10 and 22.66 mm) while the most resistant was *E. coli* with a inhibition diameters of 6-10 mm except for *Xylopia aethiopica* EO (19 mm).

Table 5 shows the result of sensitivity test of combinations against the seven strains. *Thymus vulgaris* combinations were more active than *Eucalyptus globulus* ones in term of activity spectrum. The activity of combinations is more pronounced against *Salmonella typhimurium* and *Salmonella paratyphimurium.*

Table 4: Sensitivity of strains (expressed in mm) with respect to active essential oils (EOs) and Gentamicin

	Essential oils samples						
Bacteria species	*Eu*	*Ci*	*Ct*	*Xy*	*Yl*	*Th*	Genta
E. coli	10 ± 0	6 ± 0	9 ± 0	19 ± 1	9 ± 0.67	8 ± 0.67	25.5 ± 0.58
S. typhimurium	17 ± 0.57	6 ± 0	22.66 ± 0.33	11.33 ± 0.67	14 ± 0	13 ± 0	20.5 ± 1
S. paratyphimurium	15.33 ± 0.33	6 ± 0	13 ± 0	6± 0	9 ± 0	7 ± 0	28.6 ± 0.81

***Eu** = Eucalyptus globulus**, Ci** = Citrus medica**, Ct** = Cymbopogon citratus**, Xy** = Xylopia, **Yl** =Cananga odorata**, Th** = Thymus**,** Genta = Gentamicin.*

Table 5: Sensitivity of strains (expressed in mm) according to the combinations of EO

	EOs combinations					
	Cymbopogon/ Eucalyptus			***Cymbopogon/ Thymus***		
Bacteria species	Eu_1 (2/1)	Eu_2 (1/2)	Eu_3 (1/2)	Th_1 (2/1)	Th_2 (1/2)	Th_3 (1/2)
E. coli	6 ± 0	6 ± 0	9 ± 1	7± 0	8 ± 0	8.66 ± 0.57
S. typhimurium	14.33 ± 0.57	10.33 ± 0.57	14 ± 0	15.33 ± 0.57	16.33 ± 0.57	15.33 ± 0.57
S. paratyphimurium	14.66 ± 0.57	14.33 ± 0.57	15.33 ± 0.57	13.33 ± 0.57	12.66 ± 0.57	6 ± 0

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MIB)
Antibacterial activity of essential oils singly

The MICs of single EOs were obtained ranging from 25 to 0.78 mg/mL as shown in table 6. Taking into consideration the activity and the bacteriological profile of EOs tested, only the EO of *Cymbopogon citratus* presented a bactericidal effect (MBC/MIC = 2) with MIC of 12.5 mg/mL. On the contrary, the EO of *Thymus vulgaris* presented a strong inhibition activity and bacteriostatic effect with the MIC value of 0.78 mg/mL against the sensitive strains. The rest of EOs presented bacteriostatic activity with MIC ranging 25 to 0,781 mg/mL. According to Aligianis *et al*, classification, EO activity is strong if MIC≤ 100 mg/mL; average if 100 mg/mL ≤MIC≤ 1500 mg/mL and weak if MIC≥1500mg/mL, *Thymus vulgaris* is the only essential oil of the study which got a strong inhibition effect (Aligiannis *et al.*, 2001).

The inhibition parameter of EOs (MIC) obtained in liquid medium ranged from 3.125 to 25 mg/mL as shown in table 6. Assuming that one drop of pure EO can weigh 20 to 25 mg the active EO can stop the growth of bacterial strains "*in vitro"* with one drop of active EO per mL administrated and knowing that in aromatherapy we can't administer orally more than three or four drops of EO, this result can be considered as positive (Da Silva, 2010).
Taking into consideration the activity and the bacteriological profile of EOs tested, only the EO of *Cymbopogon citratus* presented a bactericidal effect (MBC/MIC = 2) with MIC of 12.5 mg/mL. According to Vimal *et al.;* citral has a bactericidal activity against *Salmonella typhimurium*, *Escherichia coli* at 1 and 2 mg/mL (Vimal *et al.*, 2013). This bactericidal activity is linked to its high proportion in aldehyde compounds such as citral (31.4 % of neral + 46.2 % of geranial). Aldehydes act against bacterial cells by inducing the inhibition of cell proliferation through DNA and enzyme proteins binding to enhance the bacterial death (Dorman and Deans, 2000). In, *Escherichia coli, Salmonella typhimurium* and *Listeria monocitogenes*, citral permeabilizes and damages the cell membrane (Di Pasqua *et al.*, 2010; Nguefack *et al*, 2012). This could justify the bactericidal effect of its EO observed against the sensitive strains used.

On the contrary, the EO of *Thymus vulgaris* presented a strong inhibition against the sensitive strains according to Aligianis and *et al.*, (MIC ≤1.5 mg/mL) with the MIC of 0.781 mg/mL and bacteriostatic effect. This strong inhibition can be related to the main component thymol (45.5%), which in accordance to Zhou *et al.* and Vimal *et al*., have a good antibacterial activity against several strains (Zhou *et al.*, 2007; Vimal *et al.*, 2013). In fact, thymol interacts with cell membranes forming a complex with proteins by means of hydrogen bonds and hydrophobic interactions. It also impaires the citrate metabolic pathway and affects many enzymes directly or indirectly involved in synthesis of ATP (Gutierrez *et al.*, 2009). The results obtained differ from those of Kon and Rai's which showed that

the MICs of *Thymus vulgaris* EO ranged from 2.5 to 10 mg/mL against *E coli* (Kona and Rai, 2012). Knowing that this strain has not been sensitive against our EO sample, this difference in EO activity may be due to the fact that they are chemotypes of thyme EO (meaning different in chemical composition of the EOs bioactive compounds in terms of proportions). In fact, the proportions of *p*-cymene, thymol and carvacol; modulate the activity of EO varieties of *Thymus vulgaris* chemotypes (Vampa *et al*., 1988). This can explain why our sample rich in thymol (45.45%) and *p*-cymene (25%) does not present any activity against *E coli* while that of Kon and Rai's, rich in *p*-cymene (15.8%) and carvacrol (62.3%) got an activity because carvacol is more active than thymol (Dorman and Deans, 2000; Kona and Rai, 2012)**.**

Table 6: Inhibition parameters of EOs and bacterial profiles against each strain
(/): not tested; Nd = not determined.

EOs samples	**Inhibtions parameters (mg/mL)**	**Bacterial strains**		
		E. coli	***S. typhimurium***	***S. paratyphimurium***
E. globulus	CMI	12.5	12.5	6.25
	CMB	Nd	Nd	25
	CMB/CMI	Nd	Nd	4
C. medica	CMI	/	/	/
	CMB	/	/	/
	CMB/CMI	/	/	/
C. citratus	CMI	/	12.5	12.5
	CMB	/	25	25
	CMB/CMI	/	2	2
X. aethiopica	CMI	6.25	6.25	25
	CMB	25	25	Nd
	CMB/CMI	4	4	Nd
C. odorata	CMI	/	25	/
	CMB	/	Nd	/
	CMB/CMI	/	Nd	/
T. vulgaris	CMI	/	0.781	/
	CMB	/	25	/
	CMB/CMI	/	32	/
Gentamicin	CMI	0.003	0.012	0.006
	CMB	0.006	0.025	0.012
	CMB/CMI	2	2	2

Antibacterial activity of essential oils in combination

The activity of combinations was restricted to 2 bacterial strains but has been more efficient than that of individual EOs (table 7). The MIC parameters of the combined EO used individually were 12.5-0.78 mg/mL but decreased to 6.25-0.195 mg/mL showing the increase in activity in the same way. The combination especially Eu_3, Th_2 and Th_1 showed a high bactericidal effect (MBC/MIC = 2 or 1) against *Salmonella typhimurium* and *Salmonella paratyphimurium,* the strains listed during the etiological survey.

The results of EOs combinations have showed an increase in its activity because the MIC has decrease from 12.5-3.125 mg/mL to 6.5-0.195 mg/mL. This increase of efficacy could be attributed to synergism or addition effect of antibacterial compounds of the EOs combined. Some study has reported that mixture of EOs showed interaction activity with each other acting as additive, synergistic and in a few cases antagonistic agents (Gutierrez *et al.,* 2009; Bassolé *et al.,* 2010; Bassolé *et al.,* 2011). The restriction of combined EOs activities to *Salmonella typhimurium* and *Salmonella paratyphimurium* and its recurrent bactericidal activities to same strains sensitive to *Cymbopogon citratus* can be due to the fact that the whole combination activity may be influenced by the presence of *Cymbopogon citratus* EO for each one. This information can be helpful if we want to reduce the activity spectrum of an EO in order to effectively kill some target strain without killing the other strains of the intestinal micro flora. The bactericidal activity observed could be due to the bactericidal activity of *Cymbopogon citratus* EOs present in each combination. These two groups of combinations of the EOs may be used in order to provide better efficacy for treating various infections especially those due to *Salmonella* bacteria.

Table 7: Inhibition of combination parameters and bacterial profile against each stain

EOs Combinations		*S. typhimurium*	*S. paratyphimurium*
	CMI	0.781	6.250
Eu_1 (2/1)	CMB	6.520	N.F
	CMB/CMI	8	N.F
	CMI	1.562	3.125
Eu_2 (1/1)	CMB	6.250	12.5
	CMB/CMI	4	4
	CMI	1.562	3.125
Eu_3 (1/2)	CMB	3.125	6.25
	CMB/CMI	2	2
	CMI	1.562	3.125
Th_1 (2/1)	CMB	3.125	6.250
	CMB/CMI	2	2
	CMI	0.195	3.125
Th_2 (1/2)	CMB	0.195	N.F
	CMB/CMI	1	N.F
	CMI	0.195	/
Th_3 (1/2)	CMB	0.195	/
	CMB/CMI	1	/

(**/**): No tested; I/D: not determined

Conclusion

10.8% of bacterial diarrhea of children less than five years is caused by the following bacteria *E coli* and *salmonella* spp. The presence of some secondary metabolites such as hydrocarboned monoterpenes, alcohols, phenols and aldehydes in the EOs are responsible for the bacterial inhibition growth. The results of the antibacterial tests are a proof that the EOs and their combinations can be used to treat or prevent diarrheal diseases. The absolute bactericidal effect of *Thymus vulgaris* combinations (Th_2 and Th_3) against *Salmonella typhimiruim* one of the strains found during our etiological survey showed that they could be used as potential drug-Sees to indirectly reduce the symptoms of fever and diarrhea induced by *Salmonella typhimurium.* So, instead of using *Eucalyptus* combinations, we could use *Thymus* combinations where *Cymbopogon citratus* EO concentration is equal or more than that of thyme. *Thymus vulgaris* combinations are more effective on *salmonella* bacteria than those in *Eucalyptus globulus*. However, this study suggests that EOs and their combinations might be applied in aromatherapy as alternative to antibiotics during typhoid infection.

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