Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2019, 11(6):8-21

Research Article

ISSN: 0975-7384
CODEN(USA): JCPRC5

Effect of Harvest Daytime on Production, Chemical Composition and Antitrypanosomal Properties of Essential Oils of Two Species of *Cymbopogon* Growing in Benin

Florence Allanto[1], Salomé Kpoviessi[1,2,3*], Joanne Bero[2], Fernand Gbaguidi[3], Pierre Agbani[4], Bénédicta Kpadonou-Kpoviessi[1], Brice Sinsin[4] and Joëlle Quetin-Leclercq[2]

[1]Laboratory of Physics and Synthesis Organic Chemistry (LaCOPS), University of Abomey-Calavi (UAC), Faculty of Sciences and Technics (FAST), 01 BP: 4166 Cotonou, Benin

[2]Pharmacognosy Research Group, Louvain Drug Research Institute, Université catholique de Louvain, B1 7203 Av. E. Mounier 72, B-1200 Bruxelles, Belgium

[3]Laboratory of Pharmacognosy and Essential Oils (LAPHE), University of Abomey-Calavi (UAC), Faculty of health Sciences (FSS), Faculty of Sciences and Technics (FAST) 01BP: 188 Cotonou, Benin

[3]Laboratory of Applied Ecology (LEA), University of Abomey-Calavi (UAC), Faculty of Agronomic Sciences (FSA), 03 BP: 1974 Cotonou, Benin

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ABSTRACT

To investigate the effect of the harvest daytime of the Cymbopogon citratus (cd) Stapf (Cc) and Cymbopogon schoenantus (L). Spreng (Cs) leaves on the yields, composition, antitrypanosomal activity and cytotoxicity of their essential oils (EOs), volatile compounds of Cc and Cs leaves collected at three different times (7 am, 1 pm, 7 pm) on the same plants were extracted and analysed by GC/FID and GC/MS, tested on Trypanosoma brucei brucei (Tbb) and their cytotoxicity evaluated in vitro on CHO and WI38 cells.

Cs leaves contained more EO (1.88%-2.25%) than Cc ones (0.71%-0.82%). We observed qualitative and quantitative differences in the chemical composition of essential oils of Cc and Cs over the day. The main compounds were geranial, neral and β-pinene in all Cc EOs and piperitone, (+)-2-carene and elemol in all Cs EOs, regardless of the harvest daytime. This daytime variation of the chemical composition mainly influenced the antitrypanosomal activities of the Cc EOs which were more trypanocidal than Cs ones. Oils from Cc collected at 7 am and 7 pm were the most active with selectivities higher than 5 compared to WI38 but not compared to CHO, as Cc EOs were more toxic against CHO cells. These EOs needs further toxicological studies. Cs EOs were not

cytotoxic (IC_{50} >50 µg/mL), and did not show significant difference in activities between the collection times (IC_{50}=16.74-47.40 µg/mL). These activities seemed to be explained by synergy or antagonism between compounds.

Keywords: *Cymbopogon*; Essential oil production; Cytotoxicity; Antitrypanosomal; Daytime variation

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INTRODUCTION

Essential oils from plants are largely used for their aromatic, medicinal and culinary properties but several factors modify their chemical composition, impact their properties and thus limit their standardized use [1-3].

Cymbopogon citratus (DC.) Stapf (*Cc*) and *Cymbopogon schoenanthus* L. Spreng (*Cs*), are aromatic plants commonly used as food for men and cattle and as therapeutic agents. *Cc*, known as lemongrass is one of the most important plants of this genus used in several industrial areas as cosmetic, food, perfume or medicine [1]. It is currently spread throughout the world since it has ability to grow in moderate and extremely harsh climatic conditions [4]. It is commercially cultivated in many African countries, but the most important traders of this crop are from Guatemala and USA [1,5]. Moreover, its use in folk medicine enhanced its commercial value in African countries [1,2]. *Cs* is also an aromatic herb from the same genus known in Benin under the name "Susume". Fresh young leaves are used to prepare traditional meat recipes and an aromatic tea largely consumed in the North of Africa [6]. Besides its use in food, *Cs* is also used in folk medicine [7] for the treatment of several diseases [2,6,8]. Essential oils (EOs) from these species are produced for commercial purposes [4] and are especially searched for their antifungal, antimicrobial, antinociceptive, antioxidant, insecticidal, analgesic and mosquitos repellent properties [2,8-10]. Our previous study shows that the EOs of *Cc* and *Cs* from Benin possessed a strong antitrypanosomal activity against *Tbb* with a good selectivity, more active on *Tbb* than on *Plasmodium falciparum* (3D7) but EO from *Cc* also presented cytotoxicity against CHO and WI38 cells [2].

These plants are harvested all along the year and the chemical variation of their EO, due to many factors such as harvest daytime or season can lead to different industrial final products and EO properties [3,11-13]. But, to the best of our knowledge, no previous work reported the influence of the daytime of harvest on the chemical variation of the EO constituents of *Cc* and *Cs* EOs from Benin, on their antiparasitic properties and on their toxicity.

In this paper we report the chemical composition of EOs from fresh leaves of *Cc* and *Cs* collected at three sampling times (7 am, 1 pm, 7 pm) and analyzed by GC/FID and GC/MS with the aim of establishing the qualitative and quantitative changes, antitrypanosomal activities and cytotoxicities of these EOs in relation to harvest daytime.

MATERIALS AND METHODS

Plant Material

Fresh leaves of *Cymbopogon citratus* (cd) Stapf and *Cymbopogon schoenantus* (L). Spreng (Poaceae) were collected in March 2014 at three daytimes (7 am, 1 pm, 7 pm) on the same plants, from the Botanical Garden of the Abomey-Calavi University. Voucher specimens (no. AA6387 and AA6390/HNB respectively) of these leaves were conserved at the University of Abomey-Calavi Herbarium.

Chemicals and Drugs

DMEM and Ham's F12 culture media were purchased from Life technologies corporation (Grand Island, NY 14072, USA); Dulbecco's Phosphate Buffered Saline (DPBS 1X) from Invitrogen (Grand Island, NY 14072, USA);

tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide) (MTT), (S)-(+)-camptothecin, suramine, dimethyl sulfoxide (DMSO), α-pinene, β-pinene, camphene, *p*-cymene, myrcene, α-terpinene, γ-terpinene, 1,8-cineol, terpinolene, borneol, citronellyl acetate, terpine-4-ol, α-terpineol, geraniol, verbenone, carvacrol, thymol, bornyl acetate, α-copaene, β-caryophyllene, fenchone, thujone, *trans*-pinocarveol, *trans*-verbenol, lavandulol, myrtenal, *trans*-carveol, carvone, aromadendrene, *allo*-aromadendrene, γ-gurjunene, *cis*-ocimene, camphor and *n*-alkanes "C_7-C_{28}" were obtained from Sigma-Aldrich (Steinhein, Germany), Acros Organics (New jersey, USA), and Fluka Chemie (Buchs, Switzerland); α-thujene, sabinene, γ-3-carene, limonene, linalool, α-humulene, *cis*-pinane, α-phellandrene, *p*-cymenene, myrtenyl acetate and valencene were purchased from extrasynthese (Genay, France). All compounds were of analytical standard grade. Ter-butyl methyl ether (TBME) was an analytical grade solvent purchased from Fluka Chemie, and anhydrous Na_2SO_4 was of analytical reagent grade from UCB (Brussels, Belgium).

Isolation of Essential Oils

Five hundred grams (500 g) of fresh leaves were steam distillated for 3 hours in a modified Clevenger-type apparatus [14]. The extraction was carried out in triplicate. The oils were preserved in a sealed vial at 4°C. The essential oil yields were calculated based on the fresh plant material and according to previous work [2].

Chemical Analysis of Essential Oils

GC/FID and GC/MS analysis: GC/FID and GC/MS analysis were respectively carried out on a FOCUS GC (Thermo Finigan; Milan, Italy) and a TRACE GC 2000 series (Thermo-Quest, Rodano, Italy) as described in our previous work [2].

Identification of oil components: Individual components of the volatile oils were identified by comparison of their retention times with those of authentic standard references, computer matching against commercial EI-MS spectra library [15,16], home-made mass spectra library made from pure substances and components of known oils [2,17]. Mass spectrometry literature data were also used for the identification, which was confirmed by comparison of the GC retention indices (RI) on a non-polar column (determined from the retention times of a series of *n*-alkanes "C_7-C_{28}" mixture) [18]. The minimum Relative Strength Index (RSI) for MS analysis was 937. The Kovats indices (KI) calculated were in agreement with those reported by Adams [16]. Quantification (expressed as percentages) was carried out by the normalization procedure using peak areas obtained by FID. Values are expressed as mean ± standard deviation (n=3) and according to previous work [2].

Parasites, Cell Lines and Media

Trypanosoma brucei brucei strain 427 (Molteno Institute in Cambridge, UK) bloodstream forms were cultured as described [19] and as reported in our previous work [2].

The macrophage-like cell line, CHO Chinese Hamster Ovary cells (ATCC N° CCL-61, batch 4765275) and the human non cancer fibroblast cell line, WI38 (ATCC N° CCL- 75 from LGC Standards) were cultivated as described previously [2].

***In Vitro* Test for Antitrypanosomal Activity**

The *in vitro* test was performed as described by Bero et al. [20]. Suramine (a commercial antitrypanosomal drug, MP Biomedicals, Eschwege, Germany) was used as positive control in all experiments with an initial concentration

of 1 µg/mL. First stock solutions of essential oils and compounds were prepared in DMSO at 20 mg/mL. The solutions were further diluted in medium to give 0.2 mg/mL stock solutions. Essential oils and compounds were tested in eight serial threefold dilutions (final concentration range: 100-0.05 µg/mL, two wells/concentration) in 96-well microtiter plates. All tests were performed in triplicate [2].

Cytotoxicity Assay

The cytotoxicity of the oils against CHO and WI38 cells was evaluated as described by Bero et al. [21], using the tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (Sigma)) colorimetric method [22]. Camptothecin (Sigma) was used as positive cytotoxic reference compound and all experiments were made at least in duplicate as described previously [2].

Statistical Analysis

Student's t-test was used to test the significance of differences between results obtained for different samples, and between results for samples and controls (GraphPad Prism 4.0; GraphPad Software Inc., San Diego, USA). Statistical significance was set at $p<0.05$ [2].

RESULTS AND DISCUSSION

Effect of Harvest Daytime on Production of the EOs

This research was conducted to determine the diurnal variation of chemical composition, trypanocidal activities and cytotoxicity of EO from leaves of *Cc* and *Cs* collected at the same place during three different daytimes (7 am, 1 pm and 7 pm).

The EO contents (%) of leaves from *Cc* and *Cs* are presented in Table 1. These results revealed a variation of the oil yield through daytime. The two plants belong to the same genus, but showed different oil yields (<1% for *Cc* and 1.8 to 2.25% for *Cs*) that didn't present the same evolution during the day. The essential oil yield of *Cc* reached its maximum (0.82%) at 1 pm when, in the same time, that of *Cs* increased over the day (from 1.88% at 7 am) up to 2.25% at 7 pm. Statistical analysis revealed that yields obtained from *Cc* at 1 pm and at 7 pm were close and significantly higher than that from the same plant collected at 7 am. So the oil yield seems stable between 1 and 7 pm. For *Cs*, yields between 7 am and 1 pm were significantly lower than that at 7 pm. The obtained yields were close to those described by Kpoviessi et al. [2] (0.71% for *Cc* and 1.88% for *Cs*) and Tchobo et al. [23] (0.7 for *Cc*) in the same locality, Ajayi et al. [24] (0.73% for *Cc*) from South Africa and Yagi et al. [25] (2.1% for *Cs*) from Soudan. These yields are lower than those obtained by Bossou et al. [26] from Benin but their yields were calculated on the dry plant materials [27,28]. Our yields are also in accordance with the results of Khadri et al. [7] on *Cs* samples from Tunisia.

Table 1. Chemical composition and yield of EOs from *Cc* and *Cs* harvest at three daytimes (mean ± sd, n=3)

N°	Compounds[a]	bKI	Essential oils					
			Cc			Cs		
			7 am	1 pm	7 pm	7 am	1 pm	7 mp
1	α-Pinene[*h]	949	t	t	t	0.10 ± 0.00	t	t
2	β-Myrcene[*h]	993	-	-	-	0.26 ± 0.00	0.13 ± 0.00	0.17 ± 0.00
3	β-Pinene[*h]	996	10.14 ±	6.73 ±	9.26 ±	-	-	-

			0.04	0.06	0.08			
4	(+)-2-Carene[*h]	1005	-	-	-	13.05 ± 0.20	11.60 ± 0.10	13.08 ± 0.12
5	*p*-Cymene[*h]	1023	0.36 ± 0.00	0.45 ± 0.01	0.40 ± 0.01	0.49 ± 0.01	-	0.26 ± 0.02
6	Limonene*[h]	1028	0.17 ± 0.00	0.09 ± 0.01	0.24 ± 0.00	4.91 ± 0.10	4.21 ± 0.04	4.53 ± 0.05
7	(*Z*)-β-ocimene[*h]	1032	0.37 ± 0.00	0.37 ± 0.00	0.43 ± 0.00	1.00 ± 0.02	0.23 ± 0.05	0.69 ± 0.01
8	(*E*)-β-ocimene[*h]	1042	0.21 ± 0.00	0.20 ± 0.00	0.25 ± 0.00	0.68 ± 0.01	0.16 ± 0.00	0.46 ± 0.00
9	α-Terpinolene[*h]	1055	0.18 ± 0.00	0.22 ± 0.00	0.28 ± 0.00	0.21 ± 0.00	0.10 ± 0.00	-
10	Myrcenol[*o]	1092	0.42 ± 0.00	0.54 ± 0.01	0.33 ± 0.00	-	-	-
11	β-Linalool[*o]	1101	0.88 ± 0.00	1.12 ± 0.01	1.06 ± 0.01	-	-	-
12	*trans*-3(10)-caren-2-ol[*o]	1110	-	0.09 ± 0.00	0.08 ± 0.00	-	-	-
13	*trans*-β-terpineol[*o]	1111	-	-	-	1.79 ± 0.04	1.17 ± 0.01	1.31 ± 0.01
14	*cis*-β-terpineol [*o]	1120	-	0.18 ± 0.01	0.09 ± 0.01	1.17 ± 0.03	0.71 ± 0.01	0.80 ± 0.01
15	*cis*-p-mentha-2.8-dienol[*o]	1133	-	0.13 ± 0.00	0.14 ± 0.00	0.29 ± 0.02	-	-
16	α-phellandren-8-ol[*o]	1161	0.52 ± 0.00	0.55 ± 0.01	0.53 ± 0.00	0.32 ± 0.01	0.20 ± 0.00	0.21 ± 0.00
17	α-terpineol[*o]	1171	-	-	-	2.36 ± 0.02	1.24 ± 0.01	1.36 ± 0.01
18	β-Citronellal[*o]	1192	0.40 ± 0.00	0.50 ± 0.01	0.52 ± 0.00	-	-	-
19	*cis*-verbenol[*o]	1199	1.72 ± 0.01	1.86 ± 0.02	1.94 ± 0.02	0.34 ± 0.00	-	0.29 ± 0.01
20	*trans*-carane. 4.5-epoxy-[*o]	1201	2.71 ± 0.01	2.79 ± 0.03	3.09 ± 0.03	-	-	
21	*trans*-piperitol[*o]	1211	-	-	-	-	0.31 ± 0.00	0.34 ± 0.00
22	*cis*-piperitol [*o]	1230	-	-	-	0.58 ± 0.00	00.37 ± 0.00	0.41 ± 0.00
23	β-Citronellol[*o]	1244	0.35 ± 0.00	0.46 ± 0.01	0.57 ± 0.01	-	-	-
24	Neral[*o]	1268	35.44 ± 0.15	34.37 ± 0.32	34.00 ± 0.30	-	-	-
25	*cis*-geraniol[*o]	1291	4.33 ± 0.02	4.01 ± 0.04	4.46 ± 0.04	-	-	-
26	Nerol[*o]	1294	-	1.69 ± 0.02	1.49 ± 0.01	-	-	-
27	Piperitone[*o]	1296	-	-	-	59.67 ± 0.92	52.22 ± 0.45	60.52 ± 0.56
28	*p*-Mentha-1(7).8(10)-dien-9-ol[*o]	1298	-	0.40 ± 0.01	0.30 ± 0.00	-	-	-
29	Geranial[*o]	1328	39.52 ±	39.04 ±	37.25 ±	-	-	-

			0.17	0.36	0.32			
30	Nopol*[o]	1338	0.37 ± 0.00	0.68 ± 0.01	0.26 ± 0.00	-	-	-
31	β-Bourbonene[**h]	1340	0.48 ± 0.00	0.86 ± 0.01	0.34 ± 0.00	-	0.16 ± 0.00	-
32	Geranyl acetate[*o]	1344	1.05 ± 0.00	1.24 ± 0.01	1.68 ± 0.01	-	-	-
33	β-Elemene[**h]	1353	-	-	-	0.43 ± 0.01	0.84 ± 0.01	0.69 ± 0.01
34	2-Undecanone[***o]	1368	-	0.16 ± 0.00	0.13 ± 0.00	-	-	-
35	β-Caryophyllene[**h]	1394	0.17 ± 0.00	0.18 ± 0.00	0.24 ± 0.00	0.78 ± 0.01	0.81 ± 0.01	1.09 ± 0.01
36	Neric acid[*o]	1423	-	0.06 ± 0.00	-	-	-	-
37	Geranic acid[*o]	1467	-	0.23 ± 0.01	-	-	-	
38	Germacrene-D[**h]	1477	-	-	-	-	0.12 ± 0.00	-
39	β-Eudesmene[**h]	1483	-	-	-	-	0.12 ± 0.00	-
40	τ-Gurjunene[**h]	1493	-	-	-	-	0.18 ± 0.00	-
41	α-Muurolene[**h]	1499	-	-	-	t	0.08 ± 0.00	-
42	Seychellene[**h]	1505	-	-	-	0.07 ± 0.00	0.14 ± 0.00	0.09 ± 0.00
43	τ-Cadinene[**h]	1514	-	-	-	0.10 ± 0.00	0.18 ± 0.00	0.12 ± 0.00
44	α-Bergamotene[**h]	1521	0.10 ± 0.00	0.10 ± 0.00	0.16 ± 0.00	-	-	-
45	δ-Cadinene[**h]	1523	-	-	-	0.14 ± 0.00	0.37 ± 0.00	0.22 ± 0.00
46	Elemol[**o]	1556	-	-	-	4.95 ± 0.08	11.03 ± 0.10	6.80 ± 0.06
47	Geranyl butyrate[*o]	1568	-	-	-	0.37 ± 0.01	0.14 ± 0.00	0.31 ± 0.00
48	Cubenol[**o]	1579	-	-	-	t	0.18 ± 0.00	t
49	β-Caryophyllene oxide[**o]	1585	-	0.09 ± 0.00	0.07 ± 0.00	0.48 ± 0.01	0.63 ± 0.01	0.41 ± 0.00
50	Hedycaryol[**o]	1610	-	-	-	t	0.19 ± 0.00	-
51	Eudesm-7(11)-en-4-ol[**o]	1617	-	0.10 ± 0.00	0.09 ± 0.00	-	-	-
52	Guaiol[**o]	1620	-	-	-	0.15 ± 0.00	0.33 ± 0.00	t
53	τ-Eudesmol[**o]	1630	-	-	-	1.07 ± 0.02	2.27 ± 0.02	1.19 ± 0.01
54	τ-Cadinol[**o]	1639	-	-	-	0.33 ± 0.00	0.93 ± 0.01	0.37 ± 0.00
55	β-Eudesmol[**o]	1648	-	-	-	3.27 ± 0.05	7.65 ± 0.07	3.69 ± 0.03

56	Isoaromadendrene epoxide ^{**o}	1661	-	-	-	0.17 ± 0.00	0.40 ± 0.00	0.17 ± 0.00
	Total		99.89 ± 0.04	99.56 ± 0.37	99.68 ± 0.22	99.53 ± 0.06	99.40 ± 0.13	99.58 ± 0.29
	$^{\gamma}$Yield (%)		0.71 ± 0.02a	0.82 ± 0.03b	0.78 ± 0.02b	1.88 ± 0.12a	2.03 ± 0.03b	2.25 ± 0.06c

^{a}Compounds listed in order of elution from HP-5 MS column; b: Kovats indices (KI) on HP-5 MS column; Cc: Essential oil from *Cymbopogon citratus*; Cs: Essential oil from *Cymbopogon schoenantus*; *monoterpenes; **sesquiterpenes; ***non terpenes; ^{h}hydrocarbons; ^{o}oxygenated; ^{t}traces (inferior or equal to 0.05%); (-): absence or not detected; ^{g}Yield calculated based on the fresh plant material; Values are means ± standard deviation of three separate experiments. Data in the same line followed by different letters (a.b.c.…) are statistically different by Student's t-test ($p<0.05$).

Effect of Harvest Daytime on Chemical Composition of the EOs of Cc and Cs

The comparison of EOs of *Cc* and of *Cs* from South Benin, collected at various moments of the day (Table 1) confirmed qualitatively the results of our previous studies [2]. All analysed samples of *Cc* contained monoterpenes as major compounds (98.07-99.14%) along with sesquiterpenes (0.75-1.33%) and non-terpenes (less than 0.2%). For *Cs* oils, monoterpenes (72.65-87.22%) and sesquiterpenes (12.31-26.75%) were the only detected classes. Among these chemical groups, oxidized compounds (87.71-90.36% for *Cc* EO and 77.31-79.97% for *Cs* EO) were more abundant than hydrocarbon ones. Non-terpenes, the smallest chemical group, showed a percentage that varied only very slightly, according to the daytime of harvest and were characterized by the presence of undecan-2-one in *Cc* EO. Concerning monoterpenes, their percentages in *Cc* EO (99.14% at 7 am, 98.07% at 1 pm and 98.65% at 7 pm) were practically similar (not significantly different) over the daytime but the percentages of their two sub-groups varied in an opposite way (Figure 1). Monoterpene hydrocarbons percentage showed its minimal value (8.06%; significantly different from the other values of the day) at 1 pm while at the same time, oxygenated monoterpenes one rose from the morning to reach its maximum value (90.01%; significantly different from the other values of the day) at 1 pm, which compensates the loss of monoterpene hydrocarbons. Sesquiterpenes showed a percentage lower than 1.5% in all three harvest times.

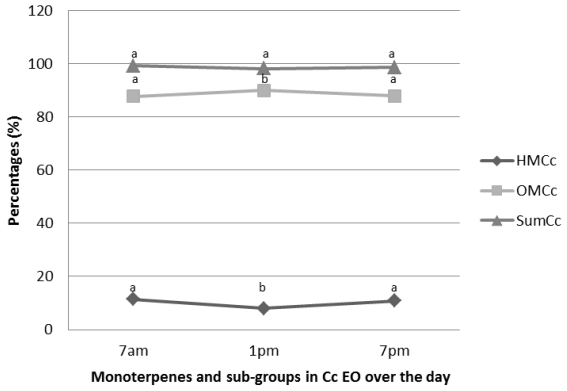

Figure 1. Diurnal variation of percentage of hydrocarbon (H) and oxygenated (O) monoterpenes (M) with their sum (Sum) in *Cc* EO over the day. Data in the same line followed by different letters ($^{a,b,\ldots}$) are statistically different by Student's t-test ($p<0.05$)

In *Cs* EO, the percentages of monoterpenes and their sub-groups followed the same trend over the day. They decreased from the morning to reach their minimal values at 1 pm (72.65%, 56.22% and 16.43% respectively for monoterpenes, oxygenated and hydrocarbon monoterpenes) before increasing at 7 pm. This trend was the contrary of what was observed for the content of sesquiterpenes and their sub-groups which were lower in the morning and rose to reach at 1 pm, their maximum values (26.75%, 23.75% and 3.00% respectively for sesquiterpenes, oxygenated and hydrocarbon sesquiterpenes) before decreasing until 7 pm. These variations compensate each other to make their sum and thus the EO yield constant over the day (Figure 2).

The nine major compounds of *Cc* EOs (percentage higher than 1%) are all monoterpenes and were geranial (**1**; 37.25%-39.52%), neral (**2**; 34.00%-35.44%), β-pinene (**3**; 6.73%-10.14%), *cis*-geraniol (**4**; 4.01%-4.46%), *trans*-carane-4,5-epoxy (**5**; 2.71%-3.09%), *cis*-verbenol (**6**; 1.72%-1.94%), nerol (**7**; 0.00%-1.69%), geranyl acetate (**8**; 1.05%-1.68%) and β-linalool (**9**; 0.88%-1.12%) (Table 1 and Figure 3). The content in each of these compounds varied according to the daytime of harvest and can be classified in four groups according to their evolution trend over the day. The percentages of **5**, **6** and **8** rose from the morning till the evening, while the percentages of **1** and **2** decreased during the day (Figure 3). This might be explained by a rapid and easy interconversion between these compounds under the sun effect [3,29,30]. The lower contents of β-pinene (**3**) and *cis*-geraniol (**4**) were observed at 1 pm while those of nerol (**7**) and β-linalool (**9**) were higher at 1 pm.

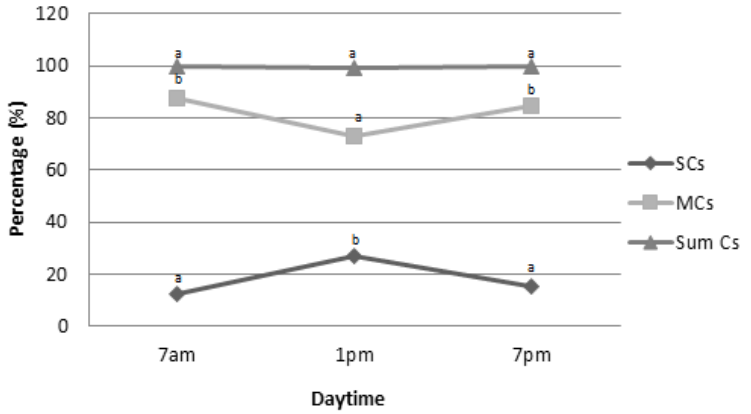

Figure 2. Diurnal variation of percentage of sesquiterpenes (S), monoterpenes (M) and their sum (Sum) in *Cc* EO over the day. Data in the same line followed by different letters ($^{a,b,\cdots}$) are statistically different by Student's t-test ($p<0.05$)

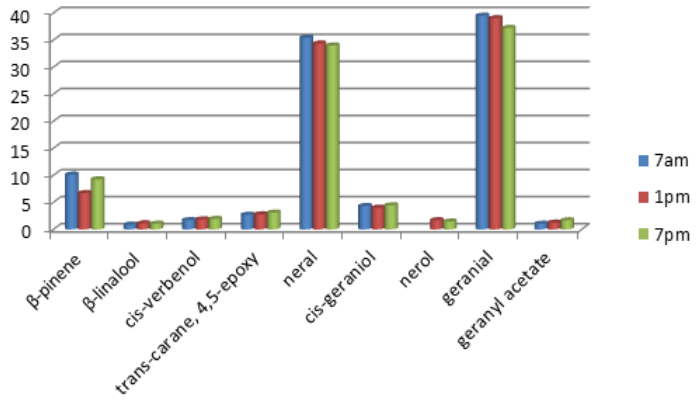

Figure 3. Diurnal variation of rate of the major components of Cc EOs.

For *Cs* EO, the major compounds belong to monoterpenes and sesquiterpenes and were piperitone (**1**; 52.22%-60.52%), (+)-2-carene (**2**; 11.60%-13.08%), elemol (**3**; 4.95%-11.03%), β-eudesmol (**4**; 3.27%-7.65%), limonene (**5**; 4.21%-4.91%), α-terpineol (**6**; 1.24%-2.36%), τ-eudesmol (**7**; 1.07%-2.27 %), *trans*-β-terpineol (**8**; 1.17%-1.79%), *cis*-β-terpineol (**9**; 0.71 %-1.17%), β-caryophyllene (**10**; 0.78%-1.09) and (Z)-β-ocimene (**11**; 0.23%-1%) (Figure 4) confirming previous results [2,26]. Lower in the morning, the percentages of major sesquiterpenes **3**, **4** and **7** rose to reach at 1 pm, their maximum values (as the general sesquiterpenes yield of this EO), before decreasing to 7 pm. This trend was opposite for the content of all the other major compounds of this EO. Furthermore, for the both oils, minor compounds composition also varies, some being at concentrations lower than the limit of detection/quantification in certain samples. These results show that for both plants, compositions vary according to the harvest time and this may modify their activities.

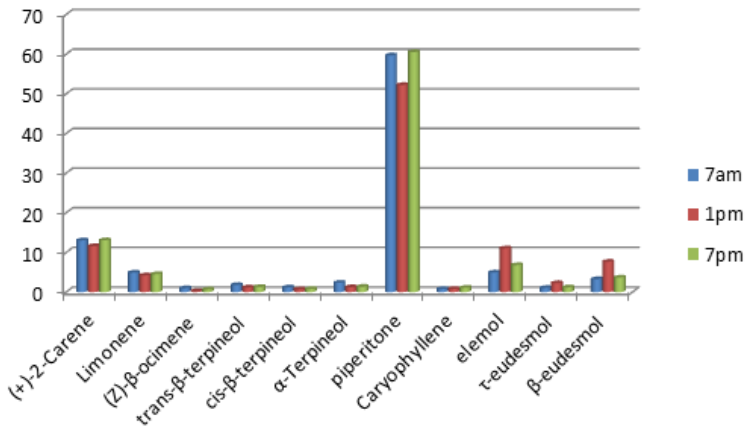

Figure 4. Diurnal variation of rate of the major components of *Cs* Eos

Effect of Harvest Daytime on Antitrypanosomal Activity and Cytotoxicity of the EOs of Cc and Cs

All the six studied EO samples were tested *in vitro* for their antitrypanosomal activities on *T. brucei brucei* and their cytotoxicity against WI38 and CHO cells. The results are summarized in Table 2 and showed that the antitrypanosomal activities of *Cc* EOs significantly varies according to the moment of harvest in the day. Other activities were not significantly different according to the harvest time, although there are differences in compositions. For antitrypanosomal activities, *Cc* EOs ($IC_{50} \leq 10.11$ µg/mL) were more actives than *Cs* ones ($IC_{50} \geq 16.74$ µg/mL), with the *Cc* samples harvested at 7 am and 7 pm being significantly more efficient ($IC_{50}<7$ µg/mL) ($p<0.05$). No significant difference was observed between *Cc* samples collected at 7 am and 7 pm. This variation of activity may be related to the variation of monoterpenes in this EO. According to the scale of Bero et al. [20], all the samples of *Cc* EO present a moderate activity on *Tbb* (2 µg/mL$\leq IC_{50} \leq$ 20 µg/mL). Concerning cytotoxicity, *Cc* EOs were more toxic on CHO cells with IC_{50} between 10.11 and 12.49 µg/mL and less toxic on WI38 cells with IC_{50} around 40 µg/mL. No significant difference between the different times of harvest was observed. *Cymbopogon citratus* being already largely used in folk medicine and cooking should need further research on its toxicity and the population sensitized about it, as selectivity indices were quite low compared to CHO cells.

The evolution trend of the antitrypanosomal activities of EO samples of *Cs* seemed different, but these differences were not significant. Concerning cytotoxicity, all *Cs* EOs had IC_{50} values higher than 50 µg/mL on the two tested cell lines showing their limited toxicity.

Selectivity indices (SI) values calculated on WI38 non cancer cells varied according to the moment of harvest in the day especially for *Cc* EOs. They were all higher than one showing some selectivity of the EOs on the parasites according to Tiuman et al. [31]. But *in vivo* studies are necessary to determine if this oil may have some interest for the treatment or prevention of sleeping sickness.

Table 2. *In vitro* antitrypanosomal activity, cytotoxicity and selectivity index of essential oils from Cc and Cs harvested at three daytimes (mean ± sd. n=3) and some of their major components

Samples		Daytimes of harvest	Antitrypanosomal activity *Tbb* (IC_{50}. µg/mL)	Cytotoxicity (IC_{50}. µg/mL)		ᵀ Selectivity Indices WI38/*Tbb*
				CHO	WI38	
Plants	Cc	7 am	6.80 ± 1.75[b]	10.63 ± 0.72[b]	39.77 ± 3.31[b]	5.85[b]
		1 pm	10.11 ± 3.30[c]	12.49 ± 2.04[b]	40.10 ± 2.58[b]	3.97[a]
		7 pm	6.24 ± 2.05[b]	10.11 ± 0.17[b]	41.15 ± 1.43[b]	6.59[b]
	Cs	7 am	16.74 ± 3.01[d]	>50	>50	>2.99
		1 pm	20.36 ± 5.92[d]	>50	>50	>2.46
		7 pm	47.40 ± 21.99[d]	>50	>50	>1.05
Compounds	*Myrcene*[€]		2.24 ± 0.27	>50	>50	>22.32
	R(+)-Limonene[€]		4.24 ± 2.27	>50	>50	>11.79
	Citral[€]		5.98 ± 0.54	20.52 ± 1.59	39.48 ± 1.59	6.6
	Citronellal[€]		2.76 ± 1.55	>50	>50	>18.12
	β-Citronellol[€]		6.45 ± 4.86	>50	>50	>7.75
	β-Pinene[€]		47.37 ± 15.65	>50	>50	>1.06
	p-Cymene[€]		76.32 ± 13.27	>50	>50	>0.66
	Nerol[€]		>100	>50	>50	<0.5
Positive control	*Suramine*		0.11 ± 0.02[a]	nd	nd	nd
	Campthotécine		nd	0.74 ± 0.09[a]	0.44 ± 0.12[a]	nd

C*c:* Essential oil from Cymbopogon citratus; C*s:* Essential oil from Cymbopogon schoenantus; WI38*:* human normal fibroblast cells; CHO*:* Chinese Hamster Ovary cells; nd*:* not determined; Tbb*:* Trypanosoma brucei brucei; IC_{50}*:* sample concentration providing 50% death of cells or parasites; ᵀSelectivity index*:* IC_{50} (WI38)/IC_{50} (Tbb); €IC_{50} values from Kpoviessi et al. [2]; Data in the same column followed by different letters (a,b,c,…) are statistically different by Student's t-test ($p<0.05$).

Correlation between Antitrypanosomal and Cytotoxic Activity and Chemical Composition of EOs of Cc and Cs

Citral, the major component of *Cc* EO (citral=neral+geranial=74.96% at 7 am, 73.41% at 1 pm and 71.25% at 7 pm) presents an IC_{50} value of 5.98 µg/mL close to the values obtained at 7 am (IC_{50}=6.8 µg/mL) and at 7 pm (IC_{50}=6.24

µg/mL) for this EO (Table 3). But the activity cannot only be explained by citral content as the less active 1 pm sample also contains high quantities of citral. This major component was also shown to be toxic against CHO cells (IC_{50}=20.62 µg/mL) and moderately toxic against WI38 cells (IC_{50}=39.48 µg/mL), explaining in part the cytotoxicity of the EO. The second major component (β-pinene=10.14% at 7 am, 6.73% at 1 pm and 9.26% at 7 am) was not toxic against these cells (IC_{50}>50 µg/mL) and had a low antitrypanosomal activity. Verbenol (1.7 to 1.9%) also showed a low activity (IC_{50}>30 µg/mL [32,33]). Minor components as β-citronellal and β-citronellol, with concentrations between 0.35 and 0.6% in the EO, showed low IC_{50} values on *Trypanosoma* (IC_{50}=2.76 µg/mL and 6.45 µg/mL respectively) with no cytotoxicity at 50 µg/mL. Thus, it is difficult to explain the observed differences in the antitrypanosomal activity by the higher or lower percentage of a particular compound, and explanation should probably be found in synergism or negative interactions between compounds, even minor ones.

Table 3. Correlation between activity and chemical components of the essential oils

Components	Diurnal variation of concentration (%) in essential oils		Antitrypanosomal activity (IC_{50}. µg/mL)	Reference
	Cc	*Cs*		
Myrcene	-	0.1-0.2	2.24 ± 0.27	[2] $^{\epsilon}$
β-pinene	6.7-10.1	-	47.37 ± 15.65	[2] $^{\epsilon}$
p-cymene	0.5-0.7	-	76.32 ± 13.27	[2] $^{\epsilon}$
Citronellal	0.4-0.6		2.76 ± 1.55	[2] $^{\epsilon}$
Limonene	-	4.3-6.4	4.24 ± 2.27	[2] $^{\epsilon}$
Citral	70.8-75.0		5.98 ± 0.54	[2] $^{\epsilon}$
β- Citronellol	0.4-0.6		6.47 ± 4.86	[2] $^{\epsilon}$
α-Pinene	t	0.1	4.09	[33,34]
Linalool	0.9-1.1	-	39.26	[33]
Piperitone	-	52.22-60.52	41.06	[36]
Aromadendrene	-	0.1-0.4	18.77	[34]
β-Caryophyllene	0.2	0.8-1.1	13.76	[35]
Verbenol	1.7-1.9		30.24	[32,33]
Caryophyllene oxide	0.1	0.4-0.7	17.67	[36]

Cc: Essential oil from *Cymbopogon citratus*; *Cs*: Essential oil from *Cymbopogon schoenantus*; $^{\epsilon}IC_{50}$ values from Kpoviessi et al. [2].

The major component of *Cs* EOs, piperitone (concentration 52.22 to 60.52%) showed an IC_{50} value of 41.06 µg/mL [36] closed to the *Cs* EOs at ($16.74 \leq IC_{50} \leq 47.40$ µg/mL). These oils contain minor compounds (such as myrcene, α-pinene, aromadendrene, β-caryophyllene and caryophyllene oxidize) with percentages (<1%) practically constant over the day that showed interesting antitrypanosomal activities (IC_{50}=2.24 µg/mL, 4.09 µg/mL [33,34], 18.77 µg/mL [33], 13.76 µg/mL [35], 17.67 µg/mL [36] respectively) but diluted by less active major compounds. As concentrations of the most active compounds remains quite unchanged, this can explain the absence of variation of antitrypanosomal activities. It has also to be noted that *Cs* EOs are not cytotoxic at the highest tested concentration.

CONCLUSION

In conclusion, the chemical composition of the essential oils of *Cc* and *Cs* from South Benin varies according to the daytime of harvest of the plants. Qualitative and quantitative differences in composition were observed despite the fact that, whatever the collection daytime, geranial, neral and β-pinene were the main components in all *Cc* oils and piperitone, (+)-2-carene and elemol in all *Cs* oils. This daytime variation of the chemical composition influenced the antitrypanosomal activities of the *Cc* EOs which were more antitrypanosomal than *Cs* ones, the most active being *Cc* EOs collected at 7 am and 7 pm. This activity may be only explained at least in part by their citral contents but synergy or antagonism may also occur. *Cc* EOs were also more cytotoxic than *Cs* ones, particularly on CHO cells, and *Cc*, already largely used in folk medicine and cooking, should need further research on its toxicity and the population sensitized about it.

This is the first report on the influence of the time of harvest of the plants on the chemical composition of the essential oil of *Cc* and *Cs* and their impact on the antitrypanosomal properties and cytotoxicity of these oils.

ACKNOWLEDGMENTS

This work was supported by the CUD (Commission Universitaire pour le Développement), CIUF (Coopération Institutionnelle Universitaire Francophone) (now ARES-CCD) and Beninese government through a grant given to S. Kpoviessi and in part by the project VALTRAMED (ARES-CCD). This work was supported by the Belgian National Fund for Scientific Research (FNRS) (T.0190.13).

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