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Chemical composition, cytotoxicity and *in vitro* antitrypanosomal and antiplasmodial activity of the essential oils of four *Cymbopogon* species from Benin ☆

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ABSTRACT

Ethnopharmacological relevance: *Cymbopogon* species are largely used in folk medicine for the treatment of many diseases some of which related to parasitical diseases as fevers and headaches. As part of our research on antiparasitic essential oils from Beninese plants, we decided to evaluate the *in vitro* antiplasmodial and antitrypanosomal activities of essential oils of four *Cymbopogon* species used in traditional medicine as well as their cytotoxicity.

Materials and methods: The essential oils of four *Cymbopogon* species *Cymbopogon citratus* (I), *Cymbopogon giganteus* (II), *Cymbopogon nardus* (III) and *Cymbopogon schoenantus* (IV) from Benin obtained by hydrodistillation were analysed by GC/MS and GC/FID and were tested *in vitro* against *Trypanosoma brucei brucei* and *Plasmodium falciparum* respectively for antitrypanosomal and antiplasmodial activities. Cytotoxicity was evaluated *in vitro* against Chinese Hamster Ovary (CHO) cells and the human non cancer fibroblast cell line (WI38) through MTT assay to evaluate the selectivity.

Results: All tested oils showed a strong antitrypanosomal activity with a good selectivity. Sample II was the most active against *Trypanosoma brucei brucei* and could be considered as a good candidate. It was less active against *Plasmodium falciparum*. Samples II, III and IV had low or no cytotoxicity, but the essential oil of *Cymbopogon citratus* (I), was toxic against CHO cells and moderately toxic against WI38 cells and needs further toxicological studies. Sample I (29 compounds) was characterised by the presence as main constituents of geranial, neral, β-pinene and *cis*-geraniol; sample II (53 compounds) by *trans*-*p*-mentha-1(7),8-dien-2-ol, *trans*-carveol, *trans*-*p*-mentha-2,8-dienol, *cis*-*p*-mentha-2,8-dienol, *cis*-*p*-mentha-1(7),8-dien-2-ol, limonene, *cis*-carveol and *cis*-carvone; sample III (28 compounds) by β-citronellal, nerol, β-citronellol, elemol and limonene and sample IV (41 compounds) by piperitone, (+)-2-carene, limonene, elemol and β-eudesmol.

Conclusions: Our study shows that essential oils of *Cymbopogon* genus can be a good source of antitrypanosomal agents. This is the first report on the activity of these essential oils against *Trypanosoma brucei brucei*, *Plasmodium falciparum* and analysis of their cytotoxicity.

1. Introduction

Aromatic plants are used since ancient times for their medicinal properties (Bakkali et al., 2008). Essential oils may be used as alternatives or adjuvants to current antiparasitic therapies and the emergence of parasites resistant to current chemotherapies highlights the importance of plant essential oils as potential novel

☆ *Chemical compounds studied in this article.*

β-Myrcene (PubChem CID: 31253); Limonene (PubChem CID: 22311); β-Citronellal (PubChem CID: 7794); Geranial (PubChem CID: 638011); Neral (PubChem CID: 643779); β-Pinene (PubChem CID: 14896); Piperitone (PubChem CID: 6987); (+)-2-Carene (PubChem CID: 78249); cis-*p*-Mentha-1(7),8-dien-2-ol (PubChem CID: 6429040); Nerol (PubChem CID: 643820).

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antiparasitic agents (Anthony et al., 2005; Moon et al., 2006; Nibret and Wink, 2010; Cheikh-Ali et al., 2011; Monzote et al., 2011; Palmeira-de-Oliveira et al., 2012).

Cymbopogon species are commonly used in folk medicine for the treatment of many diseases. *Cymbopogon citratus* is used for the treatment of nervous and gastrointestinal disturbances, and as an antispasmodic, analgesic, anti-inflammatory, anti-pyretic, diuretic and sedative (Santin et al., 2009). Decoctions of the leaves and flowers of *Cymbopogon giganteus* are used as an effective treatment against skin disorders, conjunctiva, headaches and hepatitis (Adjanohoun et al., 1979; Adjanohoun and Aké Assi, 1979, 1985). *Cymbopogon nardus* is used for cooking, perfumery, rheumatism and in the treatment of fevers, intestinal parasites and of digestive and menstrual problems (Konwar and Gohain, 1999; Abena et al., 2007). *Cymbopogon schoenathus* is used as an embrocation, a diuretic, an insecticide, an aphrodisiac, for fever, snake-bite and for the treatment of rheumatism. The smoke from the burning grass is said to dispel temporary maniacal symptoms (IUCN, 2005, Khadri et al., 2010). Essential oils of these species are known for antimicrobial, antifungal, antioxidant, analgesic, anti-nociceptive, neurobehavioral and insecticidal properties (Bassolé et al., 2011; Innsan et al., 2011, Khadri et al., 2010; Abena et al., 2007; Jirovetz et al. 2007; de Billerbeck et al., 2001; Viana et al., 2000; Onawunmia et al., 1984) and as repellent against mosquitos, the major vector of malaria (Nonviho et al., 2010; Samarasekera et al., 2006; Tyagi et al., 1998). Their direct activity against *Trypanosoma brucei* and *Plasmodium falciparum* was not very documented excepted for essential oil from *Cymbopogon nardus* of Malaysia whose *in vitro* antitrypanosomal activity was recently reported by Muhd Haffiz et al. (2013). Furthermore, they are used in traditional medicine for the treatment of symptoms given by malaria or sleeping sickness (as fevers, headaches,…). So it seemed interesting to study the antiplasmodial and antitrypanosomal activities of these essential oils and its components.

Trypanosoma brucei is the parasite responsible for human African trypanosomiasis or sleeping sickness, an illness affecting 300,000–500,000 people, while up to 60 million people in 36 countries are at risk of contracting the disease (World Health Organisation (WHO), 2002). This parasite is transmitted by the bite of infected tsetse flies of the genus Glossina.

Malaria is also a disease caused by a protozoan parasite of *Plasmodium* species and still remains a major public health problem in the world. Five hundred million people are exposed to this disease, with an annual death rate that the World Health Organisation (WHO/World Health Statistic, 2011) estimates to more than 800,000 people in 2009.

These two parasitic diseases are the cause of considerable mortality and morbidity throughout the world (WHO/World Health Statistic, 2011) and parasites develop resistance to most of the drugs used. Some of these drugs need a long course parenteral administration, show toxicity and a variable efficacy between strains or species. These reasons led to the search for new antitrypanosomal and antiplasmodial compounds and it is known that plants used in traditional medicine are a source of new leads with a new mechanism of action (Hoet et al., 2004, Bero et al., 2011).

The present study investigates the *in vitro* antitrypanosomal and antiplasmodial activity of essential oils from four plants of *Cymbopogon* genus used in traditional medicine in Benin. Oils from fresh leaves of each plant were prepared and analysed by GC/FID and GC/MS. They were evaluated for their antitrypanosomal and antiplasmodial activities and their selectivity was assessed by analysing their cytotoxicity against Chinese Hamster Ovary cells (CHO) and a human non cancer fibroblast cell line (WI38).

2. Materials and methods

2.1. Plant material

Fresh leaves of *Cymbopogon citratus* (DC) Stapf, *Cymbopogon giganteus* (Hochst.) Chiov., *Cymbopogon nardius* (L.) Rendle and *Cymbopogon schoenantus* (L.) Spreng. (Poaceae) were collected in March 2011, from the Botanical Garden of the Abomey-Calavi University. Voucher specimens (nos. AA6387, AA6388, AA6389 and 6390/HNB respectively) of these leaves were conserved at the University of Abomey-Calavi Herbarium.

2.2. 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 1 ×) from Invitrogen (Grand Island, NY 14072, USA); tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide) (MTT), (S)-(+)-camptothecin, suramine, chloroquine, artemisinin, 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 (Bruxelles, Belgium).

2.3. Isolation of essential oils

Five hundred grams (500 g) of fresh leaves were steam distillated for 3 h in a modified Clevenger-type apparatus (Bruneton, 1993). 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.

2.4. Chemical analysis of essential oils

2.4.1. GC/FID and GC/MS analysis

The GC/FID analysis was carried out on a FOCUS GC (Thermo Finigan; Milan, Italy) using the following operating conditions: HP 5MS column (30 m × 0.25 mm, film thickness: 0.25 μm) (J&W Scientific Column of Agilent Technologies, No. US167072Ã, USA); injection mode: splitless; injection volume: 1 μL (TBME solution); split flow: 10 mL/min; splitless time: 0.80 min; injector temperature: 260 °C; oven temperature was programmed as following: 50–250 °C at 6 °C/min and held at 250 °C for 5 min; the carrier gas was helium with a constant flow of 1.2 mL/min; FID detector temperature was: 260 °C. The data were recorded and treated with the ChromCard software.

The GC/MS analysis was carried out using a TRACE GC 2000 series (Thermo-Quest, Rodano, Italy), equipped with an autosampler AS2000 Thermo-Quest. The GC system was interfaced to a Trace MS mass spectrometer (ThermoQuest) operating in the electronic impact mode at 70 eV. HP 5 MS column (30 m × 0.25 mm, film thickness: 0.25 μm) was used in the same operating conditions as above. The coupling temperature of the GC was

260 °C and the temperature of the source of the electrons was 260 °C. The data were recorded and analysed with the Xcalibur 1.1 software (ThermoQuest).

2.4.2. Identification of oil components

Individual components of the volatile oils were identified by computer matching against commercial EI-MS spectra library (NIST, 1998; Adams, 1995), home-made mass spectra library obtained from pure substances and components of known oils (Kpoviessi et al., 2011). These identifications were supported by comparison of the GC retention times of a series of n-alkanes "C7–C28" mixture on a non-polar column (Kovats indices (KI)) (VanDenDool and Kratz, 1963). These indices calculated were in agreement with those reported by Adams (1995). For several compounds, comparison of data and retention times with those of authentic reference standards further confirmed the identifications. Quantification (expressed as percentages) was carried out by the normalisation procedure using peak areas obtained by FID. Values are expressed as mean ± standard deviation ($n=3$).

2.5. Parasites, cell lines and media

Trypanosoma brucei brucei strain 427 (Molteno Institute in Cambridge, UK) bloodstream forms were cultured *in vitro* in HMI9 medium containing 10% heat-inactivated foetal bovine serum (Hirumi and Hirumi, 1994).

Plasmodium falciparum chloroquine-sensitive strain 3D7 (from Prof. Grellier of Museum d'Histoire Naturelle, Paris, France) asexual erythrocytic stages were cultivated continuously *in vitro* according to the procedure described by Trager and Jensen (1976) at 37 °C and under an atmosphere of 5% CO_2, 5% O_2 and 90% N_2. The host cells were human red blood cells (A or O Rh+). The culture medium was RPMI 1640 (Gibco) containing 32 mM $NaHCO_3$, 25 mM HEPES and 2.05 mM L-glutamine. The medium was supplemented with 1.76 g/L glucose (Sigma-Aldrich), 44 mg/mL hypoxanthin (Sigma-Aldrich), 100 mg/L gentamycin (Gibco) and 10% human pooled serum (A or O Rh+). Parasites were subcultured every 3–4 days with initial conditions of 0.5% parasitaemia and 1% haematocrit.

The macrophage-like cell line, CHO Chinese Hamster Ovary cells (ATCC no. CCL-61, batch 4765275), were cultivated *in vitro* in Ham's-F12 Nutrient Mixture 21765 medium (Gibco) containing 2 mM L-glutamine supplemented with 10% heat-inactivated foetal bovine serum (Gibco), penicillin–streptomycin (100 UI/mL–100 µg/mL) and fungizone (ampotericine D 250 UG/mL). The human non cancer fibroblast cell line, WI38 (ATCC no. CCL-75 from LGC Standards) was cultivated *in vitro* in DMEM medium (Gibco) containing 4 mM L-glutamine, 1 mM sodium pyruvate supplemented with 10% heat-inactivated foetal bovine serum (Gibco), penicillin–streptomycin (100 UI/mL–100 µg/mL) and fungizone (amphotericin D 250 UG/mL).

2.6. In vitro test for antiplasmodial activity

Parasite viability was measured using parasite lactate dehydrogenase (pLDH) activity according to the method described by Makler et al. (1993). The *in vitro* test was performed as described by Murebwayire et al. (2008). Chloroquine (Sigma) or artemisinin (Sigma) were used as positive controls in all experiments with an initial concentration of 100 ng/mL. First stock solutions of essential oils and pure compounds were prepared in DMSO at 20 mg/mL. The solutions were further diluted in medium to give 2 mg/mL stock solutions. The highest concentration of solvent to which the parasites were exposed was 1%, which was shown to have no measurable effect on parasite viability. Essential oils were tested in eight serial threefold dilutions (final concentration rang: 200–0.09 µg/mL, two wells/concentration) in 96-well microtiter plates. The parasitaemia and the haematocrit were 2% and 1%, respectively. All tests were performed in triplicate.

2.7. In vitro test for antitrypanosomal activity

The *in vitro* test was performed as described by Hoet et al. (2004). 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.

2.8. Cytotoxicity assay

The cytotoxicity of the oils against CHO and WI38 cells was evaluated as described by Stevigny et al. (2002), using the tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (Sigma)) colorimetric method based on the cleavage of the reagent by dehydrogenases in viable cells (Mosmann, 1983). Camptothecin (Sigma) was used as positive cytotoxic reference compound. Stock solutions of compounds and essential oils were prepared in DMSO at 10 mg/mL. The solutions were further diluted in medium with final concentrations of 50-1.56 µg/mL (four wells/concentration). The highest concentration of solvent to which the cells were exposed was 1%, which was shown to be non-toxic. Each oil was tested in six serial twofold dilutions in 96-well microtitre plates. All experiments were made at least in duplicate.

2.9. 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 $P < 0.05$.

3. Results and discussion

3.1. Chemical composition of the essential oils

The oils extracted from fresh leaves of *Cymbopogon citratus* (I), *Cymbopogon giganteus* (II), *Cymbopogon nardus* (III) and *Cymbopogon schoenantus* (IV) collected in the same place and in the same time, were obtained with different yields (w/w) (Table 1). This may be explained by the difference between species. Moreover, these yields were less than those described by Nonviho et al. (2010) on dried leaves of three of these plants (I, II and IV), but they were calculated on the dry material (Bourkhiss et al., 2009; Nebie et al., 2011). A total of 29 (I), 53 (II), 28 (III) and 41 (IV) compounds, representing respectively 98.1% (I), 98.6% (II), 98.5% (III) and 98.6% (IV) of the hydrodistillate, were identified (Table 1). The oils were characterised by two major chemical groups: monoterpenes and sesquiterpenes, with a high amount of oxygenated monoterpenes in all studied oils (Table 2) followed by hydrocarbon monoterpenes in IV, I and II; and by oxygenated sesquiterpenes in III (Table 2). Almost all constituents of I and II were monoterpenes while III and IV contained monoterpenes with sesquiterpenes (Table 2). Non terpenic compounds were mainly identified in II, where 4,4-dimethyl androst-5-en-3-one was the major component of this class (Table 1). The sample I (29 compounds, 98.1%) obtained from *Cymbopogon citratus*

Table 1
Chemical composition and yield of essential oils from *Cymbopogon* species (mean ± standard deviation, $n=3$).

No.	Compounds[a]	KI[b]	I	II	III	IV
1	α-Pinene[c,f,i]	949	*t*	–	–	**0.1** ± 0.00
2	Camphene[c,f,i]	963	–	–	–	**0.1** ± 0.00
3	β-Myrcene[c,f,i]	993	–	t	**0.2** ± 0.00	**0.2** ± 0.00
4	β-Pinene[c,f,i]	996	**10.1** ± 0.04	–	–	–
5	(+)-2-Carene[c,f,i]	1005	–	**0.4** ± 0.00	–	**13.0** ± 0.20
6	α-Phellandrene[c,f,i]	1017	–	–	–	**0.2** ± 0.00
7	*p*-Cymene[c,f,i]	1023	**0.5** ± 0.00	–	–	–
8	Limonene[c,f,i]	1028	–	**8.3** ± 0.08	**2.2** ± 0.02	**6.4** ± 0.10
9	(*Z*)-β-ocimene[c,f,i]	1032	**0.4** ± 0.00	–	–	–
10	(*E*)-β-ocimene[c,f,i]	1042	**0.2** ± 0.00	–	–	**0.7** ± 0.01
11	Melonal[c,g]	1045	–	t	**0.2** ± 0.00	–
12	α-Terpinolene[c,f,i]	1055	**0.2** ± 0.00	**0.1** ± 0.00	–	**0.2** ± 0.00
13	Thujol[c,g,i]	1070	–	**0.1** ± 0.00	–	–
14	Fenchone-D[c,g]	1090	–	**0.3** ± 0.00	–	**0.3** ± 0.01
15	Myrcenol[c,g,i]	1092	**0.4** ± 0.00	–	–	–
16	β-Linalool[c,g,i]	1101	**0.9** ± 0.00	**0.1** ± 0.00	**0.4** ± 0.00	–
17	*trans*-3(10)-caren-2-ol[c,g]	1110	**0.1** ± 0.00	–	–	–
18	*p*-Mentha-1,3,8-trienol[c,g]	1111	–	**0.2** ± 0.00	–	–
19	*trans*-*p*-mentha-2,8-dienol[c,g]	1120	–	**15.5** ± 0.15	–	**1.8** ± 0.03
20	*cis*-*p*-mentha-2,8-dienol[c,g]	1133	**0.1** ± 0.00	**11.3** ± 0.03	–	**1.3** ± 0.02
21	Isopulegol[c,g]	1142	–	–	**0.2** ± 0.00	–
22	4-Isopropylidene-cyclohexanol[e,g]	1146	–	**0.1** ± 0.00	–	–
23	α-Phellandren-8-ol[c,g,i]	1161	**0.5** ± 0.00	**0.8** ± 0.01	–	**0.4** ± 0.01
24	*trans*-2-caren-4-ol[c,g,i]	1171	–	–	–	**0.1** ± 0.00
25	*cis*-α-terpineol[c,g,i]	1173	–	t	–	–
26	*trans*-*p*-mentha-1(7),8-dien-2-ol[c,g]	1181	–	**18.3** ± 0.17	–	**0.3** ± 0.00
27	β-Citronellal[c,g,i]	1192	**0.4** ± 0.00	t	**35.9** ± 0.34	–
28	*cis*-verbenol[c,g]	1199	**1.7** ± 0.01	–	–	–
29	*trans*-carane, 4,5-epoxy-[c,g]	1201	–	**1.5** ± 0.01	–	–
30	*cis*-*p*-mentha-1(7),8-dien-2-ol[c,g]	1206	–	**8.9** ± 0.08	–	–
31	*trans*-piperitol[c,g,i]	1211	–	–	–	**0.5** ± 0.01
32	*p*-Menth-1-en-9-al[c,g]	1215	–	**0.2** ± 0.00	–	–
33	*cis*-carveol[c,g,i]	1227	–	**7.3** ± 0.07	–	–
34	7-Methyl-3-methylene-6-octen-1-ol[e,g]	1230	–	–	–	**0.2** ± 0.00
35	β-Citronellol[c,g,i]	1244	**0.4** ± 0.00	–	**11.6** ± 0.11	–
36	*trans*-carveol[c,g]	1246	–	**17.4** ± 0.16	–	–
37	*cis*-carvone[c,g,i]	1267	–	**3.4** ± 0.03	–	–
38	Neral[c,g,i]	1268	**35.5** ± 0.15	–	**0.4** ± 0.00	–
39	Isoamyl hexanoate[e,g]	1278	–	**0.3** ± 0.00	–	–
40	*trans*-3-caren-2-ol[c,g]	1288	–	**0.2** ± 0.00	–	–
41	*cis*-geraniol[c,g,i]	1291	**4.3** ± 0.02	–	–	–
42	Perillal[c,g]	1292	–	**0.5** ± 0.01	–	–
43	Nerol[c,g,i]	1294	–	–	**24.3** ± 0.23	–
44	Piperitone[c,g,i]	1296	–	**0.1** ± 0.00	–	**60.3** ± 0.92
45	*p*-Mentha-1(7),8(10)-dien-9-ol[c,g]	1298	**0.1** ± 0.00	**0.1** ± 0.01	–	–
46	1-Methyl-2-decalone[c,g]	1304	–	**0.1** ± 0.00	–	–
47	Limonene dioxide[c,g]	1315	–	**0.1** ± 0.00	–	–
48	2-Caren-10-al[c,g]	1320	–	–	–	**0.1** ± 0.00
49	Geranial[c,g,i]	1328	**39.5** ± 0.00	–	**0.6** ± 0.01	–
50	Piperitone oxide[c,g]	1331	–	**0.1** ± 0.00	–	–
51	Exo-2-hydroxycineole acetate[c,g]	1335	–	**0.1** ± 0.00	–	–
52	Nopol[c,g]	1338	**0.4** ± 0.00	t	–	–
53	β-Bourbonene[d,f]	1340	**0.5** ± 0.00	–	–	**0.1** ± 0.00
54	Geranyl acetate[c,g]	1344	**1.0** ± 0.00	**0.1** ± 0.00	**1.3** ± 0.01	–
55	β-Elemene[d,f,i]	1353	–	t	**1.9** ± 0.02	**0.4** ± 0.01
56	2-Undecanone[d,g]	1368	**0.1** ± 0.00	–	–	–
57	3-Oxo-α-ionol[d,g]	1392	–	**0.1** ± 0.03	–	–
58	β-Caryophyllene[d,f,i]	1394	**0.2** ± 0.00	t	**0.1** ± 0.00	**0.8** ± 0.01
59	Neric acid[c,g]	1423	**0.1** ± 0.00	–	–	–
60	Isoamyl caprylate[d,g]	1427	–	**0.2** ± 0.00	–	–
61	α-Humulene[d,f,i]	1443	–	–	**0.1** ± 0.00	**0.1** ± 0.00
62	β-Cubebene[d,f]	1454	–	–	**0.1** ± 0.00	–
63	Geranic acid[c,g]	1467	**0.1** ± 0.00	–	–	–
64	α-Himachalene[d,f]	1470	–	t	–	**0.1** ± 0.00
65	Germacrene-D[d,f,i]	1477	–	–	**1.5** ± 0.01	**0.1** ± 0.00
66	β-Eudesmene[d,f,i]	1483	–	t	–	**0.1** ± 0.00
67	τ-Gurjunene[d,f]	1493	**0.1** ± 0.00	–	**0.2** ± 0.00	**0.1** ± 0.00
68	α-Muurolene[d,f]	1499	–	t	**0.2** ± 0.00	t
69	Seychellene[d,f]	1505	–	–	**0.2** ± 0.00	**0.1** ± 0.00
70	τ-Muurolene[d,f]	1514	–	t	**0.4** ± 0.00	**0.1** ± 0.00
71	α-Bergamotene[d,f]	1521	**0.1** ± 0.00	–	–	–
72	δ-Cadinene[d,f,i]	1523	–	–	**1.1** ± 0.01	**0.2** ± 0.00
73	Elemol[d,g,i]	1556	–	–	**9.0** ± 0.08	**4.9** ± 0.08
74	Geranyl butyrate[c,g]	1568	–	–	–	**0.4** ± 0.01
75	Cubenol[d,g,i]	1579	–	–	**1.8** ± 0.02	t

Table 1 (*continued*)

No.	Compounds[a]	KI[b]	I	II	III	IV
76	β-Caryophyllene oxide[d,g]	1585	**0.1** ± 0.00	**0.1** ± 0.00	–	**0.4** ± 0.01
77	Hedycaryol[d,g]	1610	–	–	–	t
78	Ledol[d,g,i]	1615	–	–	**0.6** ± 0.01	–
79	Eudesm-7(11)-en-4-ol[d,g]	1617	**0.1** ± 0.00	–	–	–
80	Phenylethyl caproate[e,g]	1619	–	**0.1** ± 0.00	–	–
81	Guaiol[d,g]	1620	–	–	–	**0.1** ± 0.00
82	τ-Eudesmol[d,g]	1630	–	–	**0.6** ± 0.01	**1.1** ± 0.02
83	τ-Cadinol[d,g,i]	1639	–	–	**1.1** ± 0.01	**0.3** ± 0.00
84	β-Eudesmol[d,g,i]	1648	–	t	–	**3.1** ± 0.05
85	α-Cadinol[d,g,i]	1650	–	–	**2.0** ± 0.02	–
86	Isoaromadendrene epoxide[d,g]	1661	–	t	–	**0.1** ± 0.00
87	(*Z*,*E*)-farnesol[d,g]	1699	–	t	**0.2** ± 0.00	**0.1** ± 0.00
88	Geranyl caproate[c,g]	1748	–	**0.1** ± 0.03	–	–
89	(−)-Spathulenol[d,g]	1779	–	t	–	–
90	Ledene alcohol[d,g]	1809	–	t	–	–
91	Phenylethyl octanoate[e,g]	1888	–	**0.1** ± 0.01	–	–
92	4,4-Dimethylandrost-5-en-3-one[e,g]	2184	–	**1.7** ± 0.02	–	–
93	Bolasterone[e,g]	2209	–	**0.2** ± 0.00	–	–
94	Norethindrone[e,g]	2336	–	**0.1** ± 0.04	–	–
	Total identified		**98.1** ± 0.41	**98.6** ± 0.97	**98.5** ± 0.92	**98.6** ± 0.52
	[h]**Yield (%)**		**0.71** ± 0.02	**0.65** ± 0.02	**1.06** ± 0.10	**1.88** ± 0.12

[a] Compounds listed in order of elution from HP-5 MS column.
[b] Kovats indices (KI) on HP-5 MS column; I=Essential oil from *Cymbopogon citratus*; II=essential oil from *Cymbopogon giganteus*; III=essential oil from *Cymbopogon nardus*; IV=essential oil from *Cymbopogon schoenantus*; t=traces (inferior or equal to 0.05%); and (−)=absence or not detected.
[c] Monoterpenes.
[d] Sesquiterpenes.
[e] Non-terpenes.
[f] Hydrocarbons.
[g] Oxygenetad.
[h] Yield calculated based on the fresh plant material.
[i] Comparison of data with reference standard.

Table 2
Chemical groups in essential oils from *Cymbopogon* species (mean ± standard deviation, $n=3$).

No.	Chemical groups	I	II	III	IV
1	Hydrocarbon monoterpenes	11.4 ± 0.04	8.8 ± 0.08	2.4 ± 0.02	20.9 ± 0.31
2	Oxygenated monoterpenes	85.5 ± 0.35	86.7 ± 0.73	74.9 ± 0.7	65.5 ± 1.01
3	**Monoterpenes**	**96.9** ± 0.39	**95.5** ± 0.81	**77.3** ± 0.09	**86.4** ± 1.32
4	Hydrocarbon sesquiterpenes	0.4 ± 0.00	*t*	5.8 ± 0.04	2.2 ± 0.02
5	Oxygenated sesquiterpenes	0.2 ± 0.00	0.3 ± 0.06	15.3 ± 0.15	10.0 ± 0.16
6	**Sesquiterpenes**	**0.6** ± 0.00	**0.3** ± 0.06	**21.1** ± 0.19	**12.2** ± 0.18
7	**Others**	**0.6** ± 0.04	**2.8** ± 0.07	–	**0.3** ± 0.00

I=Essential oil of *Cymbopogon citratus*, II=essential oil of *Cymbopogon giganteus*, III=essential oil of *Cymbopogon nardus*, IV=essential oil of *Cymbopogon schoenantus*, (−)=absence or not detected, and *t*=traces (inferior or equal to 0.05%).

was characterised by the presence as main constituents of geranial, neral, β-pinene, *cis*-geraniol, *cis*-verbenol and geranyl acetate (Table 1). The mixture of geranial and neral, two geometrical isomers constituting of citral, accounts for about 75% of the total of oil I (Table 1). Sakirigui et al. (2011) obtained 70.13% of citral, Nonviho et al. (2010) 74% and Blanco et al. (2009) 77% in *Cymbopogon citratus* oil. The sample II (53 compounds, 98.6%) obtained from *Cymbopogon giganteus* was characterised by the presence as major constituents of *trans*-*p*-mentha-1(7),8-dien-2-ol, *trans*-carveol, *trans*-*p*-mentha-2,8-dienol, *cis*-*p*-mentha-2,8-dienol, *cis*-*p*-mentha-1(7),8-dien-2-ol, limonene, *cis*-carveol, *cis*-carvone, 4,4-dimethylandrost-5-en-3-one and *trans*-carane-4,5-epoxy (Table 1). Its composition, except for limonene, approached that found in leaves from Togo (Nyamador et al., 2010). The sample III (28 compounds, 98.5%) obtained from *Cymbopogon nadus* was characterised by a high concentration of β-citronellal, nerol, β-citronellol, elemol, limonene, α-cadinol, β-elemene, cubenol, germacrene-D, geranyl acetate, δ-cadinene and τ-cadinol (Table 1). These results seem similar to those of Oliveira et al. (2011) that identified in the Brazilian species 34.61% of citronellal followed by 23.18% of geraniol and 12.10% of citronellol. The sample IV (41 compounds, 98.6%) obtained from *Cymbopogon schoenantus* showed the presence as main constituents of piperitone, (+)-2-carene, limonene, elemol, β-eudesmol, *trans*-*p*-mentha-2,8-dienol, *cis*-*p*-mentha-2,8-dienol and τ-eudesmol (Table 1). This composition was different from that described by Nonviho et al. (2010) which found 68% of piperitone in a sample from Akogbato (Benin) but was close to the results reported by Ayedoun et al. (1997) in a sample from Bassila (Benin). The major components represented over 90% of the studied oils. The concentrations of all the other constituents were less than 1.2%. Each oil was thus characterised by known compounds with percentages sometimes different from those described in the literature (Nonviho et al., 2010; Abena et al., 2007, Alitonou et al., 2006; Sidibé et al., 2001; Shahi and Tava, 1993). This variation can be due to the influence of the moment or the place of harvest in the chemical composition of these oils (Singh et al., 1994; Boruah et al., 1995; Kpoviessi et al., 2011; Kpadonou Kpoviessi et al., 2012).

Table 3
In vitro antitrypanosomal and antiplasmodial activity, cytotoxicity and selectivity index of essential oils from *Cymbopogon* species and some of their major components.

Samples	Cytotoxicity (IC_{50}, μg/mL) average ± standard deviation		Antitrypanosomal activity Tbb (IC_{50}, μg/mL) average ± standard deviation	Antiplasmodial activity *Pf* (IC_{50}, μg/mL) average ± standard deviation	[r]Selectivity indices		
	CHO	WI38			WI38/Tbb	WI38/3D7	3D7/Tbb
I	10.63 ± 0.72	39.77 ± 3.31	1.83 ± 0.13[b]	47.97 ± 13.09[c]	21.73	0.93	26.21
II	> 50	> 50	0.25 ± 0.11[a]	11.22 ± 5.35[b]	> 200	> 4.46	3.14
III	> 50	> 50	5.71 ± 1.40[c]	52.61 ± 4.79[c]	> 8.76	> 0.95	9.21
IV	> 50	> 50	2.10 ± 0.89[b]	43.15 ± 13.19[c]	> 23.81	> 1.16	20.55
Myrcene	> 50	> 50	2.24 ± 0.27[b]	nd	> 11.76		
R(+)-limonene	> 50	> 50	4.24 ± 2.27[c]	nd	> 8.94		
Citral	20.62 ± 1.59	39.48 ± 1.59	5.98 ± 0.54[c]	nd	4.01		
Citronellal	> 50	> 50	2.76 ± 1.55[b]	nd	> 7.41		
Nerol	> 50	> 50	> 100	nd	< 0.5		
β-Citronellol	> 50	> 50	6.45 ± 4..86[c]	nd	> 1.53		
β-Pinene	> 50	> 50	47.37 ± 15.65[e]	nd	> 1.06		
6-Acetoxy-*p*-mentha-1,8-diene	> 50	> 50	28.82 ± 2.91[d]	nd	> 4.64		
p-Cymene	> 50	> 50	76.32 ± 13.27[f]				
Campthotecin	0.74 ± 0.09	0.44 ± 0.12	nd	nd			
Suramine	nd	nd	0.11 ± 0.02[a]	nd			
Chloroquine	nd	nd	nd	0.02 ± 0.01[a]			
Artemisinin	nd	nd	nd	0.01 ± 0.001[a]			

I=Essential oil from *Cymbopogon citratus*, II=essential oil from *Cymbopogon giganteus*, III=essential oil from *Cymbopogon nardus*, IV=essential oil from *Cymbopogon schoenantus*, WI38=human normal fibroblast cells, CHO=Chinese Hamster Ovary cells, nd=not determined, Tbb=*Trypanosoma brucei brucei*, 3D7=chloroquine-sensitive strain of *Plasmodium falciparum*, and IC_{50}=sample concentration providing 50% death of cells or parasites. [r]Selectivity index=IC_{50} (WI38)/IC_{50} (Tbb or 3D7). Data in the same column followed by different letters ($^{a,b,c,\cdots}$) are statistically different by Student's *t*-test ($P < 0.05$). Values are means ± standard deviation of three separate experiments.

3.2. Antitrypanosomal, antiplasmodial activities and cytotoxicity

All the studied oils were tested *in vitro* for their antitrypanosomal and antiplasmodial activities respectively on *Trypanosoma brucei brucei* and *Plasmodium falciparum* 3D7 and their cytotoxicity against WI38 and CHO cells. Results are summarised in Table 3. All oils showed a stronger effect against *Trypanosoma brucei brucei* with IC_{50} values ≤ 6 μg/mL. These oils were in increased order of activity. *Cymbopogon nardus* (III, IC_{50}=5.71 ± 1.40 μg/mL), *Cymbopogon schoenantus* (IV, IC_{50}=2.10 ± 0.89 μg/mL), *Cymbopogon citratus* (I, IC_{50}=1.83 ± 0.13 μg/mL) and *Cymbopogon giganteus* (II, IC_{50}=0.25 ± 0.11 μg/mL). The student's *t*-test showed a highly significant difference between the activity of II and the other tested oils. However the activity of the essential oil from *Cymbopogon giganteus* (II) was not significantly different (*P* value > 0.1) to that of suramine (IC_{50}=0.11 ± 0.02 μg/mL) the standard compound used against this parasite. The selectivity index of the four tested oils (I=21.73; II > 200; III > 8.75 and IV > 23.81) showed that II was also the most selective. *In vivo* studies should be performed to assess its efficacy on sleeping sickness and determine if the essential oil from *Cymbopogon giganteus* (II), already used extensively in traditional medicine, can be recommended for the treatment of this illness. It will necessary to search for adequate formulation as LBDDS (lipid based drug delivery systems) (Mu et al., 2013) and to verify the absence of toxicity. Recently, Muhd Haffiz et al. (2013) reported a different IC_{50} value (0.31 ± 0.03 μg/mL) for the essential oil of *Cymbopogon nardus* from Malaysia, tested against the BS221 strain of *Trypanosoma brucei brucei*. This difference on IC_{50} value may be due to differences in the origin and the composition of the essential oil and in the strain of *Trypanosoma brucei brucei*. The antitrypanosomal activity of available major compounds of these studied oils was also evaluated. β-Myrcene (IC_{50}=2.24 μg/mL), citronellal (IC_{50}=2.76 μg/mL), R(+)-limonene (IC_{50}=4.24 μg/mL), citral (IC_{50}=5.98 μg/mL) and β-citronellol (IC_{50}=6.45 μg/mL) showed antitrypanosomal IC_{50} values near to those of studied oils and can in part explain their activities. The antitrypanosomal IC_{50} values of the other tested major components: 6-acetoxy-*p*-mentha-1,8-diene (IC_{50}=28.82 μg/mL), β-pinene (IC_{50}=47.37 μg/mL) and *p*-cymene (IC_{50}=76.32 μg/mL); and those of (±)-linalool (39.32 μg/mL), β-caryophylene (13.78 μg/mL), 1,8-cineole (83.15 μg/mL), (−)-carvone (12.94 μg/mL), piperitone (41.12 μg/mL), (−)-verbenone (30.24 μg/mL), limonene epoxide (22.58 μg/mL) and caryophyllene oxide (17.70 μg/mL) (Hoet et al., 2006; Nibret and Wink, 2010), were higher than 10 μg/mL and could not explain the strong activity of these oils but synergic effect is possible. Furthermore, most of the major constituents of II were not available and could not be tested. Even though sesquiterpenes as artemisinin are good antiparasitic agents, we note here that the two most active antitrypanosomal oils only contain trace amounts of sesquiterpenes and are particularly rich in oxygenated monoterpenes (Table 2). Two of them: *trans*-*p*-mentha-1(7),8-dien-2-ol and *trans*-carveol are major compounds of II, possessing the best activities and should be tested to elucidate their contribution to the antitrypanosomal activity of II.

Concerning the antiplasmodial activity against the chloroquino-sensitive strain of *Plasmodium falciparum* (3D7), we observed that *Cymbopogon giganteus* essential oil (II) was the only one which could be considered as having an interesting activity with an IC_{50} value ≤ 20 μg/mL, the other oils had a moderate activity (IC_{50} values between 21 and 60 μg/mL). With a selectivity index > 4.46, the essential oil *Cymbopogon giganteus* (II) can also be a good candidate for bio-guided fractionation to yield a more active fraction against *Plasmodium falciparum*. It would also be interesting to test *trans*-*p*-mentha-1(7),8-dien-2-ol and *trans*-carveol, the major compounds of this oil, on *Plasmodium*. Moreover these results showed the selectivity of the activity of the studied oils on *Trypanosoma brucei brucei* as compared to *Plasmodium falciparum* (SI > 3 for all studied oils).

The cytotoxicity tests against the Chinese Hamster Ovary (CHO) cells and the human non cancer fibroblast cell line (WI38) showed that all tested oils and components had a low cytotoxicity (IC_{50} > 50 μg/mL) (Table 2). The only exception was *Cymbopogon citratus* essential oil (I) which was toxic against CHO cells (IC_{50}=10.63 μg/mL) and moderately toxic against WI38 cells

(IC_{50} = 39.77 μg/mL). The major component of this oil (citral = neral + geranial = 74%) was also toxic against CHO cells (IC_{50} = 20.62 μg/mL) and moderately toxic against WI38 cells (IC_{50} = 39.48 μg/mL). The second major component (β-pinene = 10.10%) was not toxic against these cells (IC_{50} > 50 μg/mL).

4. Conclusions

Our study shows that the essential oils of *Cymbopogon citratus* (I), *Cymbopogon giganteus* (II), *Cymbopogon nardus* (III) and *Cymbopogon schoenantus* (IV) from Benin were more active on *Trypanosoma brucei brucei* than on *Plasmodium falciparum* (3D7). The essential oil of *Cymbopogon giganteus* (II) already used extensively in traditional medicine is the most active and could be interesting for the treatment of sleeping sickness but may also have some interest on *Plasmodium*. These oils had a low cytotoxicity except *Cymbopogon citratus* essential oil (I) which was toxic against CHO cells and moderately toxic against WI38 cells. Its major component (citral = neral + geranial) was also toxic against CHO cells and moderately toxic against WI38 cells. *Cymbopogon citratus* already largely used in folk medicine and cooking should need further research on its toxicity and the population sensitised about it. This is the first report on the activities of these essential oils against *Trypanosoma brucei brucei*, *Plasmodium falciparum* and their cytotoxicity.

Author agreement

All authors have made substantial contributions and final approval of the conceptions, drafting, and final version.

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