Vol. 13(16), pp. 384-395, September, 2019
DOI: 10.5897/JMPR2019.6791
Article Number: D243D7F61885
ISSN 1996-0875
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Journal of Medicinal Plants Research

Full Length Research Paper

Chemical composition, *in vitro* antioxidant and antiparasitic properties of the essential oils of three plants used in traditional medicine in Benin.

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Received 23 May, 2019; Accepted 8 August, 2019

***Sclerocarya birrea* (*Sb*), *Psidium guajava* (*Pg*) and *Eucalyptus camaldulensis* (*Ec*) are widely used in traditional medicine for the treatment of many diseases, some of which were related to oxidative stress and parasitic diseases. Their essential oils (EO) were analyzed by GC/MS and FID and tested in vitro for their antioxidant activities (DPPH), their anti-trypanosomal and anti-plasmodial activities against *Trypanosoma brucei brucei* (*Tbb*) (strain 427) and *Plasmodium falciparum* (*Pf*) (strain 3D7), respectively. Cytotoxicity was evaluated in vitro against CHO and WI38 cells (MTT) to evaluate the selectivity. They were shown to possess low antioxidant but a strong anti-trypanosomal and a good antiplasmodial activity with a good selectivity, except *Ec* oil whose anti-plasmodial activity was less interesting. Sb oil was the most active against *Tbb*(IC_{50} = 0.46 ± 0.28 µg/ml) and *Pf* (5.21 ± 1.12 µg/ml). All tested oils had low or no cytotoxicity against CHO and WI38 cells. GC/MS and GC/FID analysis revealed that composition of *Sb* (49 compounds) was characterised by the presence as main constituents of 7-epi-α-selinene, α-muurolene and valencene; *Pg* (60 compounds) by β-bisabolene, ar-curcumene and β-bisabolol; *Ec* (43 compounds) by *γ*-terpinene and *p*-cymene. The activity of these oils seems to be the result of a synergistic action of all their constituents, including minor ones. This study shows that essential oils of *Sb* and *Pg* can be good sources of anti-trypanosomal and anti-plasmodial agents.**

Key words: Essential oil, *S. birrea*, *P. guajava, E. camaldulensis*, antimalarial, antitrypanosomal, antioxidant.

INTRODUCTION

The emergence of parasites resistant to current chemotherapies highlights the importance of the search of potential novel anti-parasitic agents which may be used as alternatives or adjuvants to current anti-parasitic therapies (Cheikh-Ali et al., 2011; Nibret and Wink, 2010). Similarly the overproduction of free radicals in

cells induces an oxidative stress inplicated in atherosclerosis, cardiovascular diseases, hypertension, ischemia/reperfusion injury, diabetes mellitus, neuro-degenerative diseases, immuno-inflammatory and malaria (Maloueki et al., 2015; Rashid et al., 2013; Valko et al., 2007; Djordjević et al., 2008; Ayoola et al., 2008). To escape these serious consequences related to oxidative stress and parasitic diseases, the use of aromatic and medicinal plants, and especially their essential oils have been the subject of several studies (Kpoviessi et al., 2014; Safaei-Ghomi et al., 2009).

Sclerocarya birrea (A. Rich.) Hochst (Anacardiaceae), *Psidium guajava* L**.** (Myrtaceae) and *Eucalyptus camaldulensis* Dehnh (Myrtaceae) are aromatic plants used as food for men and cattle, for firewood, wood carving and in traditional medicine for many diseases (Kabiru et al., 2013; Gouwakinnou et al, 2011; Gutiérrez et al., 2008). The stem bark aqueous extract of *S. birrea* has been used to treat malaria in Benin (Gouwakinnou et al, 2011). Bark aqueous and methanolic extracts were shown by Gathirwa et al. (2008) to possess *in vitro* anti-plasmodial and *in vivo* anti-malarial efficacy alone or in combination with other medicinal plant extracts. Maceration, infusion or decoction in water of different parts of *P. guajava* are used in several countries as febrifuge or in skin problems (Gutiérrez et al., 2008; Hermans et al., 2004; Ajaiyeoba et al., 2003). Aqueous decoctions and various extracts from leaves and flowers of *P. guajava*, alone or in combination with other medicinal plant extracts possess *in vitro* anti-plasmodial activities (Kaushik et al., 2015; Tarkang et al. 2014; Rajendran et al., 2014; Chinchilla, et al., 2012). *E. camaldulensis* leaves are used alone and in combination with other plants to treat malaria and typhoid fevers in some Northern parts of Nigeria and ethanolic extracts possess *in vivo* anti-trypanosomal activities (Kabiru et al., 2013).

Essential oils of these plants are known for antimicrobial, antifungal, antioxidant, analgesic, anti-inflammatory, anti-nociceptive, antiradical, larvicidal, and insecticidal properties (Ghalem and Mohamed, 2014; Njume et al., 2011). Furthermore, these oils are used orally (drops) or by inhalation in traditional medicine for the treatment of malaria or its symptoms or sleeping sickness (Knezevic, 2016; Rasoanaivo et al., 1992; Gelfand et al., 1985).The direct activity of these essential oils against *Trypanosoma brucei* and *Plasmodium falciparum* was not very documented except for essential oil of *E. camaldulensis* from Nigeria. This oil was reported to kill in 4 mins *T. brucei brucei* parasites at a concentration of 0.4 g/ml *in vitro* (Habila et al., 2010). So, it seemed interesting to study the anti-plasmodial and anti-trypanosomal activities of these essential oils and their components.

T. brucei is the parasite responsible for human African trypanosomiasis or sleeping sickness, an illness affecting 300,000 African people, while up to 60 million people in 36 countries are at risk of contracting the disease and 6314 cases were recorded in 2013 (WHO, 2015). This parasite is transmitted by the bite of infected Tse-tse flies of the genus *Glossina*. Malaria is also a disease caused by a protozoan parasite of Plasmodium specie and still remains a major public health problem in the world. According to the latest estimates, 219 million cases of this disease occurred globally in 2017 (uncertainty range 203 to 262 million) and the disease led to 435 000 deaths (WHO, 2018).

These two parasitic diseases are the cause of considerable mortality and morbidity throughout the world and parasites develop resistance to most of the drugs used (WHO, 2018). Some of these drugs need a long course parenteral administration, show toxicity and a variable efficacy between strains or species. Free radicals also cause several diseases whose treatments are very expensive for the population. There is a need to search for new anti-trypanosomal, anti-plasmodial and antioxidant lead compounds with new mechanism of action from medicinal plants (Bero et al., 2011).

The present study aims to evaluate *in vitro* anti-trypanosomal, anti-plasmodial and antioxidant activities, along with cytotocycity against chinese hamster ovary cells (CHO) and a human non cancer fibroblast cell line (WI38) for the determination of selectivity, of essential oils from three plants: *S. birrea*, *P. guajava* and *E. camaldulensis* used in traditional medicine in Benin.

MATERIALS AND METHODS

Plant material

Fresh leaves of *S. birrea* (A. Rich.) Hochst (Anacardiaceae), *P. guajava* L. (Myrtaceae) and *E. camaldulensis* Dehnh. (Myrtaceae) were collected in March 2014, from the Botanical Garden of the Abomey-Calavi University. Voucher specimens (n°AA6384, AA6536 and AA6590/HNB respectively) were conserved at the University of Abomey-Calavi Herbarium.

Chemicals and drugs

Dulbecco's Modified Eagle Medium (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), DPPH (2,2-diphenyl-1-picrylhydrazyl), (S) - (+), ascorbic acid, (S)-(+)-camptothecin,

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suramine, chloroquine, artemisinin, dimethyl sulfoxide (DMSO) and *n*-alkanes "C_7-C_{28}" were obtained from Sigma-Aldrich (Steinhein, Germany), Acros Organics (New jersey, USA), and Fluka Chemie (Buchs, Switzerland). 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 h in a modified Clevenger-type apparatus (Bruneton, 2009). 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 (Kpoviessi et al., 2014).

Chemical analysis of essential oils

GC/MS analysis

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 5MS column (30 m × 0.25 mm, film thickness: 0.25 μm) was used; 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 to 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. 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 analyzed with the Xcalibur 1.1 software (ThermoQuest) (Kpoviessi et al., 2014).

Identification of oil components

Individual components of the volatile oils were identified by comparison with computer matching of their retention times against those of commercial EI-MS spectra library (NIST/EPA/NIH, 1998; Adams, 2007), home-made mass spectra library made from pure substances and components of known oils (Kpoviessi et al., 2011). 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) (VanDenDool and Kratz, 1963). The minimum Relative Strength Index (RSI) for MS analysis was 937. The Kovats indices (KI) calculated were in agreement with those reported by Adams (Adams, 2007). 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).

In vitro test for antioxidant activity

The DPPH method was used to evaluate the antioxidant activity of oils. In a 96-well microplate, a series of 10 successive dilutions (at 1/2) of each oil, was prepared from sample solutions at 150 µL/ml in methanol. For each concentration, three (03) tests were carried out by adding 100 µl of DPPH at 100 µg/ml in methanol at all dilutions in cascade. Thus, the DPPH was tested at a single concentration of 50 µg/ml. The plate was incubated in the dark for 20 min and the absorbance at 517 nm using a spectrophotometer. The negative control consists of 1 ml of methanolic solution and 1 ml of DPPH solution (100 µ/ml). Positive control was the solution of Ascorbic acid (1 mg/ml) (Otohinoyi et al., 2014; Brand-Williams et al., 1995)

The antiradical activity was estimated according to the following equation:

$$\%\ \text{antiradical activity} = \frac{Absorbed\ \ (negative\ \ control) - Absorbed\ \ (oil)}{Absorbed\ \ (negative\ control)} * 100$$

The extract concentration that reduces the absorbance of DPPH by 50% (EC_{50}) was obtained with the GraphPadPrism 4.0 software.

Parasites, cell lines and media

T. 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**)**. *P. 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 to 4 days with initial conditions of 0.5% parasitaemia and 1% haematocrit.

The macrophage-like cell line, CHO Chinese Hamster Ovary cells (ATCC N° 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) and penicillin–streptomycin (100 UI/mL to 100 μg/mL). The human non cancer fibroblast cell line, WI38 (ATCC N° 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) and penicillin–streptomycin (100 UI/mL to 100 μg/mL).

***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 to 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.

In vitro test for anti-trypanosomal 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 to 0.05 μg/mL, two wells/concentration) in 96-well microtiter plates. All tests were performed in triplicate.

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. 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 200 to 6.25 μg/mL. 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 fourfold dilutions in 96-well microtitre plates. All experiments were made at least in duplicate.

Statistical analysis

Student's t-test was used to test the significance of differences between sets of results 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 $P < 0.05$.

RESULTS AND DISCUSSION

Chemical composition of the essential oils

Yields (w/w) of oils extracted from fresh leaves of *Sb*, *Pg* and *Ec* (0.24, 0.78 and 1.38%, respectively) collected in the same place at the same time are given in Table 1. The yield (1.38%) of *Ec* leaves oil obtained in the present study confirms the work of Moudachirou et al. (1999) who reported the highest rate (1.30%) for this plant in Benin at Calavi in the period of February and March or at Kétou between April and May 1996. However, this yield was higher than that obtained in Morocco (0.84%) (Farah et al., 2002) and between the values 0.75 and 1.42% obtained from Tunisia (Haouel et al., 2010). For *Pg*, the yield (0.78%) was closer to that reported for this plant at Tchaada in Benin (0.82%) (Noudogbessi et al., 2013) and in Nigeria (0.75%) (Ogunwande et al., 2003) but different from the one described by Noudogbessi et al. (2013) in Missérété (0.25%) and Adjarra (0.30%) in outhern Benin. The leaves of *Sb* gave an oil yield (0.24%) in accordance with that indicated by Kpoviessi et al. (2011) for the same plant in the same area during the rainy season. These authors had also showed that this yield varies depending on the season (Kpoviessi et al., 2011). The difference between essential oil yields or chemical composition of the same plant could be explained by the influence of the location, season, and time of harvest in the day or the vegetative stage of the plant (Noudogbessi et al., 2013; Kpadonou-Kpoviessi et al., 2012; Kpoviessi et al., 2011; Moudachirou et al., 1999).

A total of 49 (*Sb*), 43 (*Ec*) and 60 (*Pg*) compounds, representing respectively 97.96% (*Sb*), 98.50% (*Ec*) and 96.10% (*Pg*) of hydrodistillate, were identified (Table 1). These oils contained more hydrocarbon compounds (60.60 to 92.55%) than oxygenated ones. Sesquiterpenes were the major terpenoids in *Sb* and *Pg* oils (95.12 and 93.81%, respectively) while *Ec* oil was characterized by the predominance of monoterpenes (96.95%) (Table 2).

The essential oil of *Sb* was characterized by the presence of 7-epi-α-selinene (37.86 ± 0.03%) of α-muurolene (25.03 ± 0.03%), and valencene (17.12 ± 0.06%) as major constituents followed by β-selinene (4.32 ± 0.01%), β-caryophyllene (3.24 ± 0.02%), epoxy-allo aromadendrene (1.54 ± 0.03%), 14-hydroxy-α-humulene (1.51 ± 0.03%) and α-copaene (1.20 ± 0.04%). The study of this oil was not very documented. Its chemical composition was close to that described by Kpoviessi et al. (2011) by GC/FID and GC/MS analysis methods.

In the *Ec* oil, γ-terpinene (57.24 ± 0.04%) predominated followed in decreasing order of rate by *p*-cymene (18.22 ± 0.02%), terpinen-4-ol (7.50 ± 0.07%), 1,8-cineole (7.49 ± 0.07%), limonene (1.82 ± 0.02%) and terpinolene (1.02 ± 0.01%). This composition was similar to that described at Calavi (Moudachirou et al., 1999) but different from those studied in Spain (Verdeguer et al., 2009), Jerusalem (Chalchat et al., 2001), Tunisia (Haouel et al., 2010), Australia, Morocco and Ivory Coast (Kanko et al., 2012), which were richer in *p*-cymene, spathulenol, cryptonne or 1, 8-cineole.

No component of the *Pg* oil exceeded a rate of 15%. Over twenty compounds exhibit a percentage higher than 1% with β-bisabolene (14.38 ± 0.03%), *ar*-curcumene (12.39 ± 0.02%), β-bisabolol (11.40 ± 0.08%) and β-caryophyllene (8.04 ± 0.03%) as major compounds. These results were more similar to those obtained in the locality of Banigbe (Benin) by Noudogbessi et al. (2013) than those obtained in other parts of the country by the same authors. Furthermore, the content of 1,8-cineole (0.44 ± 0.01%) in Benin oil was lower than that in Brazil (21.40%; with GC/MS method), Taiwan (12.40%, with GC/FID and GC/MS methods) and China (18.90% with GC/MS method) ones (Chen et al., 2007; Da Silva et al., 2003).

Anti-trypanosomal, anti-plasmodial activities and cytotoxicity

All studied oils were tested *in vitro* for their anti-trypanosomal and anti-plasmodial activities respectively on *T. brucei brucei* and *P. falciparum* 3D7 and their cytotoxicity against WI38 and CHO cells. The results are

Table 1. Chemical composition and yield of essential oils from *Sclerocarya birrea* (*Sb*), *Eucalyptus camaldulensis* (*Ec*) and *Psidium guajava* (*Pg*) (mean ± sd, n = 3).

N°	[a]Compounds	[b]IK	%*Sb*	%*Ec*	%*Pg*
1	4-hydroxy-4-methyl-pentan-2-one[&o]	835	0.19 ± 0.06	0.10 ± 0.00	-
2	α-thujene[*h]	931	0.10 ± 0.05	0.19 ± 0.00	-
3	α-pinene[*h]	939	0.09 ± 0.05	0.36 ± 0.00	-
4	camphene[*h]	953	-	tr	-
5	benzaldehyde[&o]	961			0.26 ± 0.00
6	sabinene[*h]	976	0.21 ± 0.08	0.14 ± 0.00	-
7	β-pinene[*h]	980	0.19 ± 0.10	0.31 ± 0.00	-
8	6-methylhept-5-en-2-one[&o]	985	-	-	0.17 ± 0.00
9	myrcene[*h]	991	0.10 ± 0.02	0.24 ± 0.00	
10	α-terpinene[*h]	1018	-	0.19 ± 0.00	-
11	*p*-cymene[*h]	1026	0.52 ± 0.13	18.22 ± 0.02	0.28 ± 0.00
12	limonene[*h]	1031	0.10 ± 0.01	1.82 ± 0.02	0.13 ± 0.00
13	1.8-cineole[*o]	1033	-	7.49 ± 0.07	0.44 ± 0.01
14	(*Z*)-β-ocimene[*h]	1040	-	tr	0.21 ± 0.00
15	(*E*)-β-ocimene[*h]	1050	0.20 ± 0.04	0.06 ± 0.00	0.22 ± 0.00
16	γ-terpinene[*h]	1062	tr	57.24 ± 0.04	-
17	terpinolene[*h]	1088	-	1.02 ± 0.01	-
18	*p*-cymenene[*h]	1089	-	0.10 ± 0.00	-
19	linalol[*o]	1096	0.37 ± 0.05	0.09 ± 0.00	0.13 ± 0.00
20	valerate d'isoamyle[&o]	1107	-	0.10 ± 0.00	-
21	1-methyl-4-(1-methyl propyl)-benzene[&h]	1113		-	0.17 ± 0.00
22	(*E*)-4.8-dimethyl-1.3.7-nonatriene[*h]	1113	0.10 ± 0.03	-	-
23	citronellal[*o]	1153	-	0.12 ± 0.00	-
24	verbenol[*o]	1164	-	0.06 ± 0.00	-
25	boneol[*o]	1175	-	tr	-
26	terpinene-4-ol[*o]	1182	-	7.50 ± 0.07	-
27	*p*-cymene-8-ol[*o]	1183	-	0.09 ± 0.00	-
28	α-terpineol[*o]	1196	tr	0.54 ± 0.01	-
29	(*Z*)- sabinol[*o]	1214	-	0.19 ± 0.00	-
30	isovalerate de n-hexyle[&o]	1243	-	0.10 ± 0.00	-
31	piperitone[*o]	1252	-	0.28 ± 0.00	-
32	*p*-cymene-7-ol[*o]	1287	-	0.29 ± 0.00	-
33	thymol[*o]	1298	0.13 ± 0.01	0.25 ± 0.00	0.18 ± 0.00
34	carvacrol[*o]	1298	-	0.16 ± 0.00	-
35	cyclosativene[**h]	1378	0.28 ± 0.03	-	-
36	α-copaene[**h]	1379	1.20 ± 0.04	-	1.00 ±0.02
37	β-bourbonene[**h]	1388	0.20 ± 0.01	-	-
38	β-elemene[**h]	1391	tr	-	-
39	7-epi-α-cedrene[**h]	1404	-	-	0.38 ± 0.01
40	helifolene[**h]	1406	-	-	1.13 ± 0.02
41	α-gurjunene[**h]	1409	-	0.13 ± 0.00	-
42	(*Z*)-α-bergamotene[**h]	1411	-	-	0.59 ± 0.01
43	α-cedrene[**h]	1418	-	-	1.01 ± 0.02
44	β-caryophyllene[**h]	1418	3.24 ± 0.02	-	8.04 ± 0.03
45	β-cedrene[**h]	1424	-	-	0.45 ± 0.01
46	β-copaene[**h]	1430	0.11 ± 0.04	-	-
47	β-gurjunene[**h]	1432	-	0.07 ± 0.00	-
48	(*E*)-α-bergamotene[**h]	1434	-	-	0.41 ± 0.01
49	aromadendrene[**h]	1441	0.10 ± 0.05	0.24 ± 0.00	-
50	selina-5.11-diene[**h]	1444	0.10 ± 0.05	-	-

Table 1. Contd.

51	epi-β-santalene[**h]	1446	-	-	0.19 ± 0.00
52	α-humulene[**h]	1454	0.10 ± 0.01	-	1.32 ± 0.02
53	(*E*)-β-farnesene[**h]	1458	-	0.12 ± 0.00	0.90 ± 0.01
54	β-santalene[**h]	1460	-	-	1.08 ± 0.02
55	allo-aromadendrene epoxyde[**0]	1461	-	tr	-
56	α-acoradiene[**h]	1464	-	-	2.89 ± 0.05
57	β-acoradiene[**h]	1465	-	-	0.73 ± 0.01
58	4.5-di-epi-aristochene[**h]	1470	0.21 ± 0.05	-	-
59	α-neocallitropsene[**h]	1475	-	-	1.66 ± 0.01
60	selina-4.11-diene[**h]	1475	0.44 ± 0.06	-	-
61	germacrene-D[**h]	1480	tr	-	-
62	*ar*-curcumene[**h]	1483	-	-	12.39 ± 0.02
63	β-selinene[**h]	1485	4.32 ± 0.01	-	1.23 ± 0.00
64	ledene[**h]	1491	-	0.07 ± 0.00	-
65	(*Z*)-α-bisabolene[**h]	1494	-	-	1.28 ± 0.02
66	α-selinene[**h]	1494	0.36 ± 0.20	-	1.22 ± 0.02
67	valencene[**h]	1494	17.12 ± 0.06	0.07 ± 0.00	-
68	α-zingiberene[**h]	1495	-	-	0.31 ± 0.00
69	α-muurolene[**h]	1496	25.03 ± 0.03	-	-
70	β-curcumene[**h]	1503	-	-	0.22 ± 0.00
71	β-bisabolene[**h]	1509	-	-	14.38 ± 0.03
72	γ-cadinene[**h]	1510	-	tr	0.45 ± 0.00
73	β-sesquiphellandrene[**h]	1516	-	-	3.02 ± 0.05
74	δ-cadinene[**h]	1520	-	tr	0.60 ± 0.01
75	(*E*)-γ-bisabolene[**h]	1521	-	-	2.07 ± 0.03
76	7-epi-α-selinene[**h]	1522	37.86 ± 0.03	-	-
77	(*E*)-α-bisabolene[**h]	1530	-	-	0.64 ± 0.01
78	Selina-3.7(11)-diene[**h]	1557	0.27 ± 0.40	-	-
79	(*E*)-nerolidol[**O]	1564	-	-	2.38 ± 0.04
80	viridiflorol[**O]	1564	-	0.06 ± 0.00	-
81	ar-tumerol[**O]	1578	-	-	0.70 ± 0.01
82	caryophyllene oxyde [**O]	1581	0.06 ± 0.04	-	2.20 ± 0.03
83	β-copaen-4-α-ol[**O]	1587	-	-	0.11±0.00
84	globulol[**O]	1595	-	0.25± 0.01	-
85	guaïol[**O]	1607	-	-	0.75±0.01
86	humulene-1.2-epoxyde[**O]	1608	-	-	-
87	epi-globulol[**O]	1612	-	-	0.82±0.01
88	humulene epoxyde-D[**O]	1616	-	-	0.25±0.00
89	1.10-diepi-cubenol[**O]	1619	-	-	0.22±0.00
90	epi-cubenol[**O]	1627	0.07 ± 0.10	-	1.07±0.02
91	α-acorenol[**O]	1629	-	-	0.21±0.00
92	γ-eudesmol[**O]	1632	0.13 ± 0.10	0.05 ± 0.00	-
93	β-acorenol[**O]	1634	-	-	2.21 ± 0.03
94	gossonorol[**O]	1638	-	-	1.50 ± 0.02
95	allo-aromadendrene epoxyde[**O]	1640	1.54 ± 0.03	-	-
96	epi-α-muurolol[**O]	1641	0.10 ± 0.01	-	0.30 ± 0.00
97	α-muurolol[**O]	1646	0.08 ± 0.10	-	0.80 ± 0.01
98	α-eudesmol[**O]	1652	-	0.14 ± 0.00	0.50±0.01
99	α-cadinol[**O]	1654	0.16 ± 0.10	-	2.20±0.03
100	selin-11-en-4-α-ol[**O]	1660	0.23 ± 0.03	-	2.00±0.03
101	intermedeol[**O]	1667	0.22 ± 0.01	-	-
102	β-bisabolol[**O]	1671	-	-	11.40 ± 0.08

Table 1. Contd.

103	nerolidyl acetate**O	1675	-	-	0.80±0.01
104	α-bisabolol**O	1683	-	-	3.40 ± 0.06
105	(2*Z*.6*Z*)-farnesol**O	1694	-	-	0.10±0.00
106	(2*Z*.6*E*)-farnesol**O	1712	-	-	0.20±0.00
107	14-hydroxy-α-humulene**O	1714	1.51 ± 0.03	-	-
108	(2*E*.6*E*)-farnesol**O	1753	-	-	0.10±0.00
109	benzyl benzoate &o	1777	-	-	0.10±0.00
110	nootkatone**O	1800	0.08 ± 0.01	-	-
111	phtalates&o	1852	0.12 ± 0.02	-	-
112	acide hexadecanoïque&o	1951	0.09 ± 0.01	-	-
113	phytol***O	2097	0.33 ± 0.01	0.05 ± 0.00	-
	Total		97.96±0.06	98.50±0.03	96.10±0.02
	γYield (%)		0.24±0.01(a)	1.38±0.02(c)	0.78±0.02(b)

[a] Compounds listed in order of elution from HP-5 MS column; [b]= Kovats indices (KI) on HP-5 MS column; * = monoterpenes; *Sb* = Essential oil from *S. birrea*; *Ec* = Essential oil from *E. camaldulensis*; *Pg* = Essential oil from *P. guajava*; ** = sesquiterpenes; *** = diterpene; & = non terpenes; h = hydrocarbons; o = oxygenated; t = traces (inferior or equal to 0.05%); (-) = absence or not detected; γYield calculated based on the fresh plant material; Values are means±standard deviation of three separate experiments.

Table 2. Chemical groups of essential oils from *Sclerocarya birrea* (*Sb*). *Eucalyptus camaldulensis* (*Ec*) and *Psidium guajava* (*Pg*) (mean ± sd. n = 3).

N°	Chemical groups	%*Sb*	%*Ec*	%*Pg*
1	Hydrocarbon compounds	92.55 ±1.60	80.59 ±0.09	60.60 ±0.44
2	Oxygenated compounds	5.41 ±0.71	17.91 ±0.16	35.50 ±0.41
3	Hydrocarbon monoterpenes	1.61 ±0.51	79.89 ±0.09	0.84 ±0.00
4	Oxygenated monoterpenes	0.50 ±0.06	17.06 ±0.15	0.75±0.01
5	Monoterpenes	2.11 ±0.57	96.95 ±0.24	1.59 ±0.01
6	Hydrocarbon sesquiterpenes	90.94 ±1.09	0.70 ±0.00	59.59 ±0.44
7	Oxygenated sesquiterpenes	4.18 ±0.56	0.50 ±0.01	34.22 ±0.40
8	Sesquiterpenes	95.12 ±1.65	1.20 ±0.01	93.81 ±0.84
9	Diterpenes	0.33 ±0.01	0.05 ±0.00	-
10	Others	0.40 ±0.09	0.30 ±0.00	0.70±0.00

Sb = Essential oil from *S. birrea*; *Ec* = Essential oil from *E. camaldulensis*; *Pg* = Essential oil from *P. guajava*; (-) = absence or not detected; Values are means±standard deviation of three separate experiments, calculated from the individual percentages of the components.

summarized in Table 3.

These oils show an interesting anti-trypanosomal activity, the most interesting being *Pg* (IC_{50} = 1.16 ± 0.16 μg/ml) and *Sb* (IC_{50} = 0.46 ± 0.28 μg/ml). According to Bero et al. (2014), Ec oil has a moderate anti-trypanosomal activity (2 ≤ IC_{50} ≤ 20 μg/ml). While the other oils exhibited good activities (IC_{50} ≤ 2 μg/ml) and could be of interest for future development (Bero et al., 2014). The activity of *Sb* oil was not significantly different (P value = 0.1628> 0.1) than that of suramin (IC_{50} = 0.11 ± 0.02 μg/ml), the standard compound used against this parasite. The selectivity index of the three tested oils (*Sb* = 79; *Ec* > 19 and *Pg* = 33) showed that *Sb* 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 *Sb* already consumed by livestock and extensively used in traditional medicine, can be recommended for the treatment of this illness. It will be necessary to search for adequate formulation as LBDDS (lipid based drug delivery systems) (Mu et al., 2013) and to verify the absence of toxicity. To our knowledge, this is the first report of the activity of the essential oil of these three plants from Benin on *T. brucei brucei* except Habila et al. (2010) who showed that a concentration of 0.4 g/ml of Ec oil from Nigeria killed *T. brucei brucei* parasites in 4 min. Essential oils of plants from the same family (Myrtaceae) as *Leptospermum scoparium* Forst., *Melaleuca alternifolia*, *Syzygium aromaticum* (L.) Merr

Table 3. *In vitro* antitrypanosomal, antiplasmodial and antioxidant activity, cytotoxicity and selectivity index of essential oils from *S. birrea* (*Sb*), *E. camaldulensis* (*Ec*) and *P. guajava* (*Pg*) (mean ± sd. n = 3) and some of their major components.

Sample	Antioxydant activity (IC_{50}, µg/ml) Average ± standard deviation	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	[T]Selectivity indices		
		CHO	WI38			WI38/Tbb	WI38/3D7	3D7/Tbb
Sb	5106	31.19±1.80	36.17±3.31	0.46±0.28[a]	5.21±1.12[b]	78.6	6.9	11.3
Ec	9510	>50	>50	2.65±0.48b[b]	51.30±4.35[d]	>18.9	> 1.0	19.4
Pg	19290	39.00±0.80	38.00±2.00	1.16±0.16[b]	12.02±2.99[c]	32.8	3.2	10.4
myrcene[€]	-	>50	>50	2.24±0.27[b]	nd	>22.3	-	-
R(+)-limonene[€]	-	>50	>50	4.24±2.27[c]	nd	>11.8	-	-
citronellal[€]	-	>50	>50	2.76±1.55[b]	nd	>18.1	-	-
β-pinene[€]	-	>50	>50	47.37±15.65[e]	nd	>1.1	-	-
p-cymene[€]	-	>50	>50	76.32±13.27[f]	-	-	-	-
Campthotecin	-	0.74±0.09	0.44±0.12	nd	nd	-	-	-
Ascorbic acid	20	nd	nd	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]	-	-	-

Sb = Essential oil from *S. birrea*; *Ec* = Essential oil from *E. camaldulensis*; *Pg* = Essential oil from *P. guajava*; WI38 = human normal fibroblast cells; CHO = Chinese Hamster Ovary cells; nd = not determined; *Tbb* = *Trypanosoma brucei brucei*; 3D7 = Chloroquine-sensitive strain of *Plasmodium falciparum*; IC_{50} = sample concentration providing 50% death of cells or parasites; [T]Selectivity index = IC_{50} (WI38) / IC_{50} (*Tbb* or 3D7); [€]IC_{50} values from Kpoviessi et al., 2014; Data in the same column followed by different letters ($^{a,b,c,\cdots}$) are statistically different by Student's t-test ($P < 0.05$).

and L. M. Perry Cheel and *Kunzea ericoides* (A. Rich) Joy Thomps, showed anti-trypanosomal activities, respectively with IC_{50} values of 16.90, 0.50, 1.90 and 13.60 µg/ml (Bero et al., 2014). It was also reported that the ethanolic extract of *Pg* leaf was able to produce alterations in the biochemical parameters in the kidney and liver of rats experimentally infected with *T. brucei brucei* (Adeyemi and Akanji, 2011).

Concerning the anti-plasmodial activity against the chloroquine-sensitive 3D7 *P. falciparum* strain, *Sb* (IC_{50} = 5.21 ± 1.12 µg/ml) and *Pg* (IC_{50} = 12.02 ± 2.99 µg/ml) essential oils showed moderate activity with $2 \leq IC_{50} \leq 20$ µg/ml. The *Ec* (IC_{50} = 51.30 ± 4.35 µg/ml) oil was less interesting against this parasite. *Sb* aqueous extract in combination with three other medicinal plants was reported to exhibit high malaria parasite suppression (chemo-suppression >90%) *in vivo* with a doses of 100 mg/kg/d at different ratios tested interperitoneally or per os (Gathirwa et al., 2008). Recently, promising *in vitro* antiplasmodial activity against 3D7 ($IC_{50} \leq 20$ µg/ml), was seen in leaves ethyl acetate extracts and methanol extracts of *Pg* (Kaushik et al., 2015) and synergistic activities of combination with ethanol and water macerations of *Mangifera indica*, *Carica papaya*, *Cymbopogon citratus*, *Citrus sinensis*, and *Ocimum gratissimum* were reported against *P. falciparum* 3D7 and Dd2 strains (Tarkang et al., 2014). Aqueous decoctions of *Pg* also showed anti-plasmodial activity against chloroquine resistant *P. berghei* (Rajendran et al., 2014).

With a selectivity index > 6 and 3, respectively, the essential oils of *Sb* and *Pg* can also be good candidates for bio-guided fractionation to yield a more active and less toxic fraction against *P. falciparum*. These results may explain, at least in part the use of these plants in the treatment of malaria in Benin (Gouwakinnou et al., 2011; Hermans et al., 2004). Moreover, these results indicate the selectivity of the activity of the studied oils on *T. brucei brucei* as compared to *P. falciparum* (SI > 10 for all studied oils). The cytotoxicity tests against the Chinese Hamster

Table 4. Correlation between activity and chemical components of the essential oils.

Compound	Concentration (%) in essential oils			Antitrypanosomal activity (IC_{50}. µg/ml)	Reference
	Sb	*Ec*	*Pg*		
Myrcene	0.10 ±0.02	0.24 ±0.00	-	2.24 ±0.27	Kpoviessi et al. (2014)[&]
β-pinene	0.19 ±0.10	0.31 ±0.00	-	47.37 ±15.65	Kpoviessi et al. (2014)[&]
p-cymene	0.52 ±0.13	18.22 ±0.02	0.28 ±0.00	76.32 ±13.27	Kpoviessi et al. (2014)[&]
Citronellal	-	0.12 ±0.00	-	2.76 ±1.55	Kpoviessi et al. (2014)[&]
Limonene	0.10 ±0.01	1.82 ±0.02	0.13 ±0.00	4.24 ±2.27	Kpoviessi et al. (2014)[&]
α-pinene	0.09 ±0.05	0.36 ±0.00	-	4.09	Bero et al. (2014)
Sabinene	0.21 ±0.08	0.14 ±0.00	-	17.67	Bero et al. (2014)
1.8-cineole	-	7.49 ±0.07	0.44 ±0.01	83.02	Bero et al. (2014)
γ-terpinene	tr	57.24±0.04		136.91	Bero et al. (2014)
Linalol	0.37±0.05	0.09±0.00	0.13±0.00	39.26	Bero et al. (2014)
Terpinen-4-ol	-	7.50±0.07	-	39.51	Nibret and Wink (2010)
Piperitone	-	0.28±0.00	-	41.06	Nibret and Wink (2010)
Thymol	0.13±0.01	0.25±0.00	0.18±0.00	22.83	Bero et al. (2014)
Carvacrol	-	0.16±0.00	-	11.23	Bero et al. (2014)
α-cedrene	-	-	1.01±0.02	4.06	Nibret and Wink, (2010)
Aromadendrene	0.10±0.05	0.24±0.00	-	18.77	Bero et al. (2014)
β-caryophylene	3.24±0.02	-	8.04±0.03	13.76	Bero et al. (2014)
(*E*)-nerolidol	-	-	2.38±0.04	1.70	Bero et al. (2014)
caryophyllene oxyde	0.06±0.04	-	2.20±0.03	17.67	Nibret and Wink (2010)

Sb = Essential oil from *S. birrea*, *Ec* = Essential oil from *E. camaldulensis*, *Pg* = Essential oil from *P. guajava*, [&]values were previously published (Kpoviessi et al., 2014)

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} > 31 µg/ml) (Table 3).

Correlation of activity and chemical composition of essential oils

The antitrypanosomal activity of available major compounds of these studied oils was also evaluated or obtained from literature (Table 4).

The essential oil of *Sb* contains active compounds as myrcene (IC_{50} = 2.24 µg/ml), limonene (IC_{50} = 4.24 µg/ml), α-pinene (IC_{50} = 4.09 µg/ml), while sabinene (IC_{50} = 17.67 µg/ml), aromadendrene (IC_{50} = 18.77 µg/ml), β-caryophyllene (IC_{50} = 13.76 µg/ml) and caryophyllene oxide (IC_{50} = 17.67 µg/ml) had moderate activity. Other compounds as β-pinene (IC_{50} = 47.37 µg/ml), *p*-cymene (IC_{50} = 76.32 µg/ml), linalool (IC_{50} = 39.26 µg/ml) and thymol (IC_{50} = 22.83 µg/ml) had low activities (Bero et al., 2014). All these compounds do not explain the interesting activity (IC_{50} = 0.46 µg/ml) of this essential oil. The absence of described activity for not available and major compounds as 7-epi-α-selinene (37.86 ± 0.03%), α-muurolene (25.03 ± 0.03%), and valencene (17.12 ± 0.06%) could not help to explain this activity.

The first four major constituents of the essential oil of *Ec*: γ-terpinene (57.24%; IC_{50} = 136.91 µg/ml), *p*-cymene (18.22%; IC_{50} = 76.32 µg/ml), 1,8-cineole (7.49%; IC_{50} = 83.02 µg/ml) and terpinen-4-ol (7.50%; IC_{50} = 39.51 µg/ml) have very low anti-trypanosomal activities with IC_{50} values > 20 µg/ml which could not explain the moderate activity (IC_{50} = 2.65 µg/ml) observed for this oil. Furthermore, the oil contains minor compounds showing activity close to the activity of the crude oil with IC_{50} values < 5 µg/ml. Indeed, myrcene, citronellal and α-pinene with a concentration lower than 1%, showed IC_{50} values of 2.24 µg/ml; 2.76 µg/ml and 4.09 µg/ml respectively. Limonene (1.82%) had also a low IC_{50} value (IC_{50} = 4.24 µg/ml). These components seemed to act synergistically in the oil. These results confirm those obtained by Kpoviessi et al. (2014) that described the possibility of synergy effect in the essential oil of *Cymbopogon* spp.

No compound in *Pg* oil does exceed the concentration of 15%. β-caryophyllene (8.04 ± 0.03%), the fourth compound of this oil, showed moderate activity (IC_{50} = 13.76 µg/ml), while caryophyllene oxide (2.20%; IC_{50} = 17.67 µg/ml), α-cedrene (1.01%; IC_{50} = 4.06 µg/ml) and limonene (0.13%; IC_{50} = 4.24 µg/ml) were more effective. (*E*)–nerolidol (2.38 %) showed an interesting activity with an IC_{50} value of 1.70 µg/ml (Bero et al., 2014) similar to that (IC_{50}=1.16 µg/ml) obtained from the oil. Furthermore,

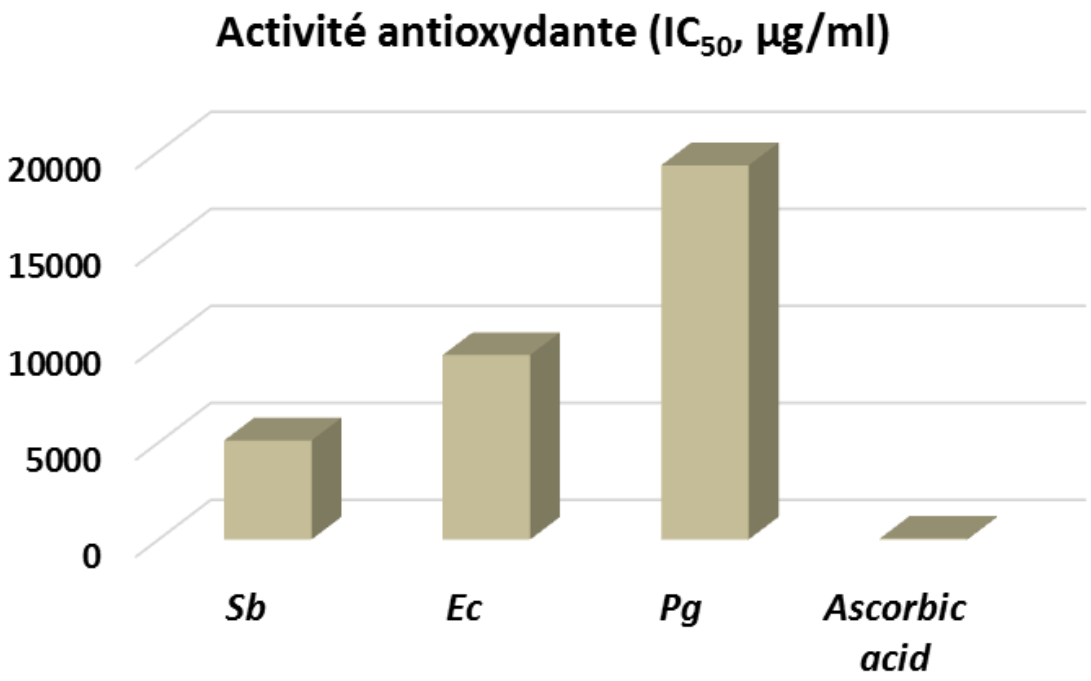

Figure 1. Comparison of the antioxidant activity of essential oils and ascorbic acid.
Sb = Essential oil from *S. birrea*, *Ec* = Essential oil from *E. camaldulensis*, *Pg* = Essential oil from *P. guajava* ,

β-bisabolene (14.38 ± 0.03%), *ar*-curcumene (12.39 ± 0.02%) and β-bisabolol (11.40 ± 0.08%), the three first major constituents of this oil, were not available and could not be tested. Recently, bisabolol oxide derivatives from *Artemisia persica* ethyl acetate extracts, exhibited *in vitro* antimalarial activity against *P. falciparum*, with IC_{50} values ranging from 1.14 to 7.92 µg/ml (Moradi-Afrapoli et al., 2013).

Given the activity observed for pure compounds, these essential oils seem to be the result of a synergistic action of all its constituents, including minor ones.

Antioxidant activity

The antioxidant activity of the studied oils was expressed in IC_{50} values and recorded in Table 3. The essential oil of *Sb* (IC_{50} = 5106 µg/ml) showed the highest activity, followed by that of *Ec* (IC_{50} = 9510 µg/ml) and by that of *Pg* (IC_{50} = 19290 µg/ml). The studied oils were all active, but less than ascorbic acid (IC_{50} = 20 µg/ml), the reference compound used in the test (Figure 1). This activity is quite low and could be explained by the presence in these oils of some high active components as β-caryophyllene (IC_{50} = 3.68 µ/ml; Pujiarti et al., 2012) and some less active components as *β*-pinene (IC_{50} = 20.05± 0.03 µg/ml; Kazemi, 2015); *p*-cymene (IC_{50} = 20.05± 0.4 µg/ml; Kazemi, 2015). Safaei-Ghomi et al. (2009), showed that the antioxidant activity of the major components tested separately gives lower results compared to the activity of the whole components of the essential oil. Furthermore, presence of allylic compounds and / or benzyls (less than 1% in the studied oils; Table 2) could contribute to this activity. Therefore, the antioxidant activity of our oils could be explained by a synergy of action between their different constituents (Safaei-Ghomi et al., 2009; Vardar-Unlu et al., 2003).

Conclusion

Our study shows that the essential oils of *S. birrea*, *E. camaldulensis* and *P. guajava* from Benin were more active on *T. brucei brucei* than on *Plasmodium falciparum* (3D7) and very weakly antioxidants. The essential oils of *S. birrea* and *P. guajava* already used extensively in traditional medicine and consumed by livestock were the most active and could be interesting for the treatment of sleeping sickness but may also have some interest on *Plasmodium*. These plants contain components with low, moderate or very good activities, which appear to act synergistically in their essential oils. These oils had a low cytotoxicity against CHO and WI38 cells. This is the first report on the activities of these essential oils against *T. brucei brucei*, *P. falciparum* and their cytotoxicity.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interest.

ABBREVIATIONS

Sb: *Sclerocarya birrea*, **Pg:** *Psidium guajava,* **Ec:** *Eucalyptus camaldulensis.*

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