



ELSEVIER

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jep

Research Paper

In vitro antitrypanosomal and antiplasmodial activities of crude extracts and essential oils of *Ocimum gratissimum* Linn from Benin and influence of vegetative stage



Bénédicta G.H. Kpadonou Kpoviessi^{a,b}, Salomé D.S. Kpoviessi^{a,c}, Eléonore Yayi Ladekan^b, Fernand Gbaguidi^b, Michel Frédérick^d, Mansourou Moudachirou^b, Joëlle Quetin-Leclercq^c, Georges C. Accrombessi^a, Joanne Bero^{c,*}

^a Laboratory of Physic and Synthesis Organic Chemistry (LaCOPS), University of Abomey-Calavi, Faculty of Sciences and Technics (FAST), BP: 4521, Cotonou, Benin

^b National Laboratory of Pharmacognosy, Benin center of Scientific and Technical Research (CBRST), BP 06 Oganla, Porto-Novo, Benin

^c Pharmacognosy Research Group, Louvain Drug Research Institute, Université catholique de Louvain, B1.72.03 Av. E. Mounier 72, B-1200 Bruxelles, Belgium

^d Université de Liège, Drug Research Center, Laboratoire de Pharmacognosie, Av. de l'Hôpital 1, B36, B-4000 Liège, Belgium

ARTICLE INFO

Article history:

Received 7 May 2014

Received in revised form

23 June 2014

Accepted 8 July 2014

Available online 21 July 2014

Keywords:

Ocimum gratissimum

Essential oils

Crude extracts

Antitrypanosomal activity

Antiplasmodial activity

Cytotoxicity

ABSTRACT

Ethnopharmacological relevance: Different parts of *Ocimum gratissimum* Linn are largely used in folk medicine for the treatment of many diseases, some of which related to parasitological infections as fevers and headaches. In order to validate their use and to clarify the plant part which possesses the best antiparasitic properties, we decided to evaluate the *in vitro* antiplasmodial and antitrypanosomal activities of essential oils and crude extracts from leaves, stems and seeds of *Ocimum gratissimum* as well as their cytotoxicity.

Materials and methods: The essential oils and ethanol crude extracts of leaves and stems of *Ocimum gratissimum* from Benin, were obtained in pre and full flowering stages. Seeds obtained only in full flowering stage, were also extracted. The oils were isolated by hydrodistillation and analyzed by GC/MS and GC/FID. Extracts and essential oils were tested *in vitro* against *Trypanosoma brucei* and *Plasmodium falciparum*. 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 and toxicity was assessed against *Artemia salina* Leach.

Results: The essential oils and non-volatile crude extracts of *Ocimum gratissimum* were more active on *Trypanosoma brucei* than on *Plasmodium falciparum* (3D7). This activity varies according to the vegetative stage (pre and full flowering) and the plant part (seeds, stems and leaves) extracted. The best growth inhibition of *Trypanosoma brucei* was observed with ethanol crude extracts of leaves ($IC_{50} = 1.66 \pm 0.48 \mu\text{g/mL}$) and seeds ($IC_{50} = 1.29 \pm 0.42 \mu\text{g/mL}$) in full flowering stage with good selectivity ($SI > 10$). The chemical composition of the essential oil from aerial parts (47 compounds), characterized by the presence as main constituents of *p*-cymene, thymol, γ -terpinene, β -myrcene and α -thujene, depends on the vegetative stage. The oil contained some minor compounds such as myrcene ($IC_{50} = 2.24 \pm 0.27 \mu\text{g/mL}$), citronellal ($IC_{50} = 2.76 \pm 1.55 \mu\text{g/mL}$), limonene ($IC_{50} = 4.24 \pm 2.27 \mu\text{g/mL}$), with good antitrypanosomal activities. These oils and crude extracts were not toxic against *Artemia salina* Leach and had a low cytotoxicity except leaves and seeds ethanol extracts obtained in full flowering which showed toxicity against CHO and WI38 cells.

Conclusions: Our study shows that ethanol crude extracts of leaves and seeds of *Ocimum gratissimum* in full flowering stage can be a good source of antitrypanosomal agents. This is the first report about the relation between the plant part extracted, the vegetative stage of the plant, the antitrypanosomal and antiplasmodial activities and the cytotoxicity of essential oils and non-volatile extracts of *Ocimum gratissimum* from Benin.

© 2014 Elsevier Ireland Ltd. All rights reserved.

* Corresponding author. Tel.: +32 27647292; fax: +32 27647293.

E-mail address: joanne.bero@uclouvain.be (J. Bero).

1. Introduction

From South-East of Asia, *Ocimum gratissimum* Linn is a perennial and odoriferous shrub currently present on all continents and whose therapeutic virtues are universally recognized (Sulistiarini, 1999; Prabhu et al., 2009). In Africa, its therapeutic potential is extremely broad and varies according to the countries (Adjadohoun and Ake Assi, 1979). In Cameroun, plant's infusions are considered tonic and pectoral, juice of its sheets are used to relieve headaches, giddiness, cold and cough (Mapi, 1988). In Ivory Coast, ophthalmias, ear infections and dermatoses are treated with various preparations of this plant (Bissangou, 1993). In Nigeria, Oliver (1960) prescribed it in diarrhoeas treatment whereas Sofowora (1970) indicated it for the respiratory affections and as anthelmintic. It was also used for headache, fever, ophthalmic and skin diseases, and pneumonia (Onajobi, 1986; Ilori et al., 1996). The same virtue was recognized in Rwanda. In Togo, plant's infusion is antitussive; juice of its fresh leaves is antiarrheic and antidiarrheic; its aqueous maceration is used in hematuries and purulent urethritis treatment (Adjadohoun, 1986 and 1989). In Benin, the aqueous maceration of its pulp or aerial parts is used in dystopias, pelvic pains, digestive dysmenorrhoeas, colics, candidoses, vomiting, hemorrhoid and diarrhea. Decoction of stems is used in the treatment of hepatitis, cough, asthma and wounds infections (Adjadohoun, 1986; Ayedoun, 1995). The juice of the leaves is used in anginas, cephalgias, headache, fever and malnutrition. The plant inflorescences are used in the composition of many foods as aromatizing (Adjadohoun, 1989). The fresh leaves are used as a laxative, while its infusion serves as a relief for headaches, fever, diarrhea, dysentery, pile and convulsion (Danziel, 1996). It is also usually sold on the markets for its condimental properties. Fresh aerial parts are directly consumed as vegetable in traditional soups and dried and powdered aerial parts are used in many traditional foods (Yayi, 1998). The essential oil is also used as aromatizing or adjuncts to soups, tea and several meals in Benin and in some African countries (Yayi et al., 2004).

Essential oils of *Ocimum gratissimum* Linn from several countries such as Brazil, Nigeria, Ivory Coast, Cameroon, Kenya, Togo were shown to have antimicrobial properties (Dubey et al., 1997; Nakamura et al., 1999; Adebolu and Salau, 2005; Akinyemi et al., 2005; Janine et al., 2005; Mbata and Saikia, 2005; Orafiya et al., 2006; Terezinha et al., 2006; Matasyoh, et al., 2007; Oussou et al., 2010). Recently, we studied the influence of the daytime of harvest and vegetative stages on essential oil constituents of *Ocimum gratissimum* Linn from Benin, and its antimicrobial properties (Kpadonou Kpoviessi et al., 2012) and showed the antimicrobial activities of its non volatile extracts and fractions (Kpadonou Kpoviessi et al., 2013). As shown before, the plant was used for the treatment of many diseases some of which related to parasitical diseases as fevers and headaches (Onajobi, 1986; Danziel, 1996; Ilori et al., 1996). In Kenya, the leaves decoction was orally used by Digo community for traditional treatment of malaria (Nguta et al., 2011), the whole plant extract from Congo (Tona et al., 2007), the leaves extract from Nigeria (Abiodun et al., 2011) and South India (Kamaraj et al., 2012) show antiplasmodial activity, but to our knowledge the antiparasitic activity of the plant remains poorly documented (Tchoumboungang et al., 2005; Kaou et al., 2008; Adamu et al., 2009; Oliveira et al., 2009; Borges et al., 2012; Musuyu Muganza et al., 2012) and no previous work was published on the influence of the vegetative stages on the antiparasitic properties of *Ocimum gratissimum* Linn from Benin and on the evaluation of its cytotoxicity.

As the entire aerial part of the plant is consumed and in order to clarify the best moment of harvest and plant part which possesses the best antiparasitic activities and reduced toxicity, we evaluated the influence of the vegetative stages and plant part

extracted on antitrypanosomal and antiplasmodial properties of essential oils and crude extracts of *Ocimum gratissimum* Linn from Benin, and evaluated their cytotoxicity against two mammalian cell lines and their toxicity against *Artemia salina* Leach.

2. Materials and methods

2.1. 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, chloroquine, artemisinin, dimethyl sulfoxide (DMSO), α -pinene, β -pinene, camphene, p-cymene, myrcene, α -terpinene, γ -terpinene, 1,8-cineol, terpinolene, borneol, citronellol 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 "C7-C28" were obtained from Sigma-Aldrich (Steinheim, 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 Na₂SO₄ was of analytical reagent grade from UCB (Bruxelles, Belgium). The eggs of *Artemia salina* Leach were obtained from JBL society (JBL GmbH&Co.KG, Germany).

2.2. Vegetable material collection:

Aerial parts of *Ocimum gratissimum* Linn were collected (dry season) in Abomey-Calavi at Mariagleta (south of the Republic of Benin) at various vegetative stages (pre and full flowering) at 7 am and identified by the National Herbarium of the University of Abomey-Calavi (Benin). Vouchers specimens (no. AA6381/HNB) were deposited at the National Herbarium of the University.

2.3. Extraction of crude extract

Seeds, leaves and stems of *Ocimum gratissimum* Linn collected at pre and full flowering stages were separately dried and reduced to powder. The powdered plant materials (100 g) was mixed with 1 L of ethanol for 72 h at room temperature and percolated at 1 mL/min (total solvent volume used was 2 L). After evaporation under reduced pressure, the ethanol extracts were dried in an oven at 45 °C to remove traces of solvent. Each extraction was triplicated. Each dried extract was kept in a freezer before biological tests.

2.4. Isolation of essential oils

Extraction of fresh aerial parts was carried out by hydrodistillation during 2 h with an improved Clevenger type apparatus (Clevenger, 1928; Bruneton, 1993) and dried with anhydrous Na₂SO₄. The oils were preserved in a light-free cold room, for GC/FID and GC/MS analysis and biological tests. Yields were calculated according to the fresh vegetable mass.

2.5. Chemical analysis and constituents identification of essential oils

The analysis of the essential oils was performed by GC/FID and GC/MS (AFNOR, 2000) and relative retention times on three GC columns (Sadler Research Laboratories, 1986).

GC/FID analysis: The GC analysis was firstly carried out on a DELS/IGC 121C equipped with a CP WAX 52 CB (25 m × 0.3 mm, df: 0.25 μm) column, an injector (split/splitless) and a flame ionization detector (FID). The temperatures of the injector and the detector were respectively 240 °C and 250 °C. The following temperatures programming of the oven was: isothermal at 50 °C for 5 min followed by 2 °C/min gradient until 220 °C. Carrier gas was nitrogen with a flow rate of 1 ml/min, injection used the split mode (1/10). Combustible gas was hydrogen and oxidizing gas was air with respectively a flow rate of 30 ml/min and 300 ml/min. The volume of oil injected was 0.5 μl. A Hewlett Packard 5890 equipped with flame ionization detector (FID) and split/splitless injector was secondly used for compound separation with a glass capillary column (30 m × 0.25 mm i.d.) coated with DB 5 phase thickness 0.25 μm or glass capillary column (30 m × 0.25 mm) coated with Supel cowax (0.20 μm film thickness). The other conditions were identical.

GC/MS: The oils were analyzed on a Hewlett Packard gas chromatograph model 6890 coupled to a Hewlett-Packard MS Model 5873 equipped with an HP5 column (30 m × 0.25 mm, df: 0.25 μm) programmed from 50 °C (5 min) to 300 °C at 5 °C/min, and 5 min hold. The carrier gas was helium (1 ml/min), injection used the split mode (1/10); injector and detector temperatures were 250 °C and 320 °C respectively. The MS ran in electron impact at 70 eV, electron multiplier 2200 V, ion source temperature 230 °C. Mass spectral data were acquired in the scan mode in the *m/z* range 33–450.

The oil constituents were identified by matching their mass spectra and retention indices with reference libraries (Masada, 1967; Sadler Research Laboratories, 1986; Sandra and Bicchi, 1987; McLafferty and Stauffer, 1991; NIST, 1994; Adams, 1995; Heneberg et al., 1995) and according to previous work (Kpadonou Kpoviessi et al., 2012).

2.6. 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 fetal 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) and as described previously (Kpoviessi et al., 2014).

The macrophage-like cell line, CHO Chinese Hamster Ovary cells (ATCC Number CCL-61, batch 4765275) and the human non cancer fibroblast cell line, WI38 (ATCC Number CCL-75 from LGC Standards) were cultivated as described previously (Kpoviessi et al., 2014).

2.7. 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) and in our previous work (Kpoviessi et al., 2014).

2.8. In vitro test for antitrypanosomal activity

The *in vitro* test was performed as described by Hoet et al. (2004) and in our previous work (Kpoviessi et al., 2014).

2.9. Toxicity test against *Artemia salina* Leach.

The test was performed against *Artemia salina* Leach by the method of Michael et al. (1956) summarized by Vanhaecke et al. (1981) and by Sleet and Brendel (1983) and as previously reported (Kpadonou Kpoviessi et al., 2012). Camptothecin (Sigma) was used as positive reference compound.

2.10. Cytotoxicity assay

The cytotoxicity of the oils, extracts and pure compounds against CHO and WI38 cells was evaluated as described by Stevigny et al. (2002) and as reported in our previous work (Kpoviessi et al., 2014).

2.11. 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 $P < 0.05$ (SAS/STAT, 1990; Steel and Torrie, 1980).

3. Results and discussion

3.1. Comparison of yields of ethanol crude extracts and essential oils

The ethanol crude extracts yields varied between 1.97% and 4.81%, depending on the vegetative stage and the plant part (Table 1). With the same extraction procedure, leaves produced more extract (4.81% in pre-flowering and 3.44% in full flowering) than seeds (2.66%) and stems (2.06% in full flowering and 1.97% in pre-flowering). For essential oil, the pre-flowering plant gives slightly less oil ($\approx 0.71\%$) than the full flowering one ($\approx 0.78\%$). These yields (0.71–0.78%) (Table 2) range between 0.4% and 0.8% as found in previous results (Yayi et al., 2004). Recent works showed variation of oil yield depending on the vegetative stage and the daytime (Kpadonou Kpoviessi et al., 2012).

3.2. Variation of the chemical composition of the essential oil

GC/MS and GC/FID analysis of essential oils provided data on the chemical compositions (Table 2) which, qualitatively, confirmed previous works (Yayi et al., 2004; Kpadonou Kpoviessi et al., 2012).

Table 1

Yields obtained for ethanol crude extracts (mean ± sd, $n=3$).

Samples	Pre-flowering		Full flowering		
	Leaves	Stems	Leaves	Stems	Seeds
Yields (%) (g/100 g)	4.81 ± 0.33 ^c	1.97 ± 0.05 ^a	3.44 ± 0.39 ^b	2.06 ± 0.12 ^a	2.66 ± 0.32 ^{ab}

*Means followed by the same letter (a ou b) are not significantly different at 5% level according to Newman–Keuls' test.

Table 2
Chemical composition of essential oils of aerial part of *Ocimum gratissimum* Linn (mean \pm sd, n=3).

Compounds	KI	Percentage (%)			
		Pre-flowering I		Full flowering I	
Aliphatic compounds		0.18	\pm 0.01	0.16	\pm 0.00
octan-3-one	987	0.18	\pm 0.01	0.16	\pm 0.00
Monoterpenes		88.83	\pm 0.61	92.15	\pm 0.51
Hydrocarboned monoterpenes		55.87	\pm 0.26	56.72	\pm 0.30
α-thujene	927	3.37	\pm 0.02	3.86	\pm 0.05
α -pinene	933	0.88	\pm 0.00	0.95	\pm 0.02
camphene	948	0.11	\pm 0.00	0.12	\pm 0.00
sabinene	973	0.93	\pm 0.01	0.89	\pm 0.00
β -pinene	976	0.52	\pm 0.00	0.41	\pm 0.00
β-myrcene	991	4.24	\pm 0.03	4.53	\pm 0.02
α -phellandrene	1003	0.15	\pm 0.00	0.22	\pm 0.00
δ -3-carene	1011	0.19	\pm 0.00	0.22	\pm 0.00
α -terpinene	1017	1.93	\pm 0.02	2.35	\pm 0.03
<i>p</i>-cymene	1027	31.53	\pm 0.11	28.08	\pm 0.06
limonene	1029	1.43	\pm 0.01	1.53	\pm 0.01
β -phellandrene	1031	0.34	\pm 0.00	0.44	\pm 0.00
(<i>Z</i>)- β -ocimene	1038	0.27	\pm 0.01	0.13	\pm 0.00
(<i>E</i>)- β -ocimene	1048	0.13	\pm 0.00	0.05	\pm 0.00
γ-terpinene	1069	7.69	\pm 0.05	10.91	\pm 0.10
terpinolene	1088	0.16	\pm 0.00	0.16	\pm 0.00
<i>p</i> -cymenene	1090	1.88	\pm 0.00	1.76	\pm 0.01
<i>p</i> -menthane-1,3,8-triene	1111	0.12	\pm 0.00	0.11	\pm 0.00
Oxygenated monoterpenes		32.96	\pm 0.35	35.43	\pm 0.21
1,8-cineol	1034	0.21	\pm 0.00	0.09	\pm 0.00
<i>Cis</i> -sabinene hydrate	1069	1.33	\pm 0.01	1.22	\pm 0.02
Linalol	1096	0.33	\pm 0.00	0.16	\pm 0.00
<i>trans</i> -sabinene hydrate	1098	0.36	\pm 0.00	0.33	\pm 0.00
<i>Trans</i> -thujone	1118	0.25	\pm 0.01	0.22	\pm 0.00
citronellal	1154	0.15	\pm 0.00	0.06	\pm 0.03
umbellulone	1171	0.09	\pm 0.00	0.07	\pm 0.00
borneol	1172	0.44	\pm 0.02	0.69	\pm 0.00
terpinen-4-ol	1183	2.1	\pm 0.10	1.71	\pm 0.02
<i>p</i> -cymen-8-ol	1189	0.43	\pm 0.00	0.25	\pm 0.00
α -terpineol	1192	0.3	\pm 0.00	0.17	\pm 0.00
thymol methyl ether	1238	0.43	\pm 0.01	0.48	\pm 0.01
estragol	1274	0.14	\pm 0.00	0.09	\pm 0.00
<i>p</i> -cymen-7-ol	1288	0.14	\pm 0.00	0.09	\pm 0.00
Thymol	1311	25.43	\pm 0.20	29.13	\pm 0.13
carvacrol	1315	0.83	\pm 0.00	0.67	\pm 0.00
Sesquiterpenes		10.46	\pm 0.07	7.22	\pm 0.06
Hydrocarboned sesquiterpenes		9.25	\pm 0.06	6.33	\pm 0.04
α -copaene	1380	0.12	\pm 0.00	0.06	\pm 0.00
β -elemene	1398	0.29	\pm 0.01	0.15	\pm 0.00
β -caryophyllene	1429	2.93	\pm 0.02	2.43	\pm 0.01
α - <i>trans</i> -bergamotene	1433	0.34	\pm 0.00	0.15	\pm 0.01
α -humulene	1463	0.43	\pm 0.00	0.31	\pm 0.00
germacrene D	1483	0.2	\pm 0.00	0.41	\pm 0.00
γ -selinene	1485	0.19	\pm 0.00	-	
β -selinene	1491	3.41	\pm 0.01	2.01	\pm 0.02
α -selinene	1494	1.06	\pm 0.02	0.63	\pm 0.00
7- <i>epi</i> - α -selinene	1528	0.28	\pm 0.00	0.18	\pm 0.00
oxygenated sesquiterpenes		1.21	\pm 0.01	0.89	\pm 0.02
caryophyllene oxide	1596	1.11	\pm 0.01	0.82	\pm 0.01
1,2-epoxydehumulene	1622	0.1	\pm 0.00	0.07	\pm 0.01
Total		99.47	\pm 0.69	99.53	\pm 0.57
*Yield (%)		0.71	\pm 0.01	0.78	\pm 0.01

I=Essential oil from aerial part of *Ocimum gratissimum* collected at 7 a.m.

* Yield calculated on base of the fresh plant material (mean \pm sd, n=3).

All analyzed samples contained monoterpenes (88.83–92.15%), sesquiterpenes (7.22–10.46%) and aliphatic compounds (0.16–0.18%) among which hydrocarbon compounds were more abundant than oxidized compounds, with a total of 47 constituents identified. The aliphatic compounds are the weakest chemical group. Their percentages varied very little according to the vegetative stage (0.15–0.18%). Monoterpenes are the major group with a percentage of 88.83% in pre-flowering stage that increases at about 3% in the full flowering stage. Hydrocarbon monoterpenes

are almost equal in the two studied stages (55.87% and 56.72% respectively in pre and full flowering) while oxygenated monoterpenes are slightly less abundant in pre-flowering stage (32.96%) than in the full flowering one (35.43%). The five major compounds of all the analyzed samples were found in this chemical group (Table 2, Fig. 1) which were in the order of decreasing percentage: *p*-cymene (28.08–31.53%), thymol (25.43–29.13%), γ -terpinene (7.69–10.91%), β -myrcene (4.24–4.53%) and α -thujene (3.37–3.86%). Among these compounds, *p*-cymene and thymol constituted

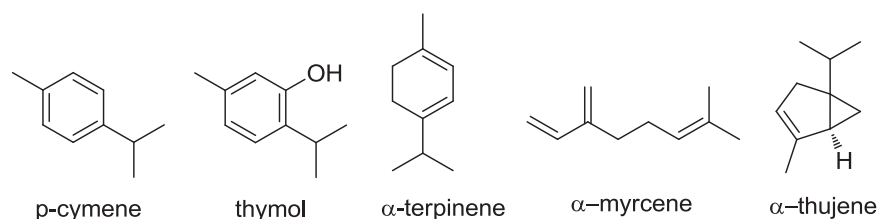


Fig. 1. Chemical structures of the five major compounds found in the essential oils.

together more than 50% of the oils. The percentages of *p*-cymene, thymol and γ -terpinene varied over the vegetative stage while those of β -myrcene and α -thujene seemed constant. This may have an effect on their antiparasitic activities.

In addition, the percentage of sesquiterpenes also varied according to the vegetative stages. Contrarily to the monoterpenes, the highest percentage of sesquiterpenes (10.46%) was obtained in the pre flowering stage and it decreases of about 3% in the full flowering stage.

3.3. Impact of the vegetative stage on the antitrypanosomal and antiplasmodial activities of the essential oils and the crude extracts of *Ocimum gratissimum*

All the studied oils and crude extracts were tested *in vitro* for their antitrypanosomal, antiplasmodial, toxic and cytotoxic activities summarized in Table 3. All oils showed a weak effect against both parasites with IC_{50} values $\geq 27 \mu\text{g/mL}$ and the oil obtained in full flowering stage was the most active. It was more active against *Trypanosoma brucei brucei* ($IC_{50}=27.23 \pm 3.74 \mu\text{g/mL}$) than against *Plasmodium falciparum* 3D7 ($IC_{50}=49.29 \pm 12.35 \mu\text{g/mL}$). The antitrypanosomal activity of available major compounds of these studied oils was previously evaluated (Kpoviessi et al., 2014). The moderate activities observed with the oils were confirmed by those of their two major compounds: thymol ($IC_{50}=22.86 \mu\text{g/mL}$) (Nibret and Wink, 2010) and *p*-cymene ($IC_{50}=76.32 \mu\text{g/mL}$). The differences on IC_{50} values observed between the oils obtained in pre-flowering ($IC_{50}=69.59 \pm 7.33 \mu\text{g/mL}$) and in full flowering ($IC_{50}=27.23 \pm 3.74 \mu\text{g/mL}$) stages could be partially explained by the lower concentration of thymol and higher concentration of *p*-cymene in pre-flowering stage (25.43% and 31.53% respectively) than in full-flowering (29.13% and 28.08% respectively) and by the variation of the concentration of non-major compounds as β -myrcene ($IC_{50}=2.24 \pm 0.27 \mu\text{g/mL}$), citronellal ($IC_{50}=2.76 \pm 1.55 \mu\text{g/mL}$) and R(+)-limonene ($IC_{50}=4.24 \pm 1.27 \mu\text{g/mL}$) which showed strong antitrypanosomal activities ($IC_{50} \leq 5 \mu\text{g/mL}$) with good selectivity ($SI > 10$).

The essential oils of *Ocimum gratissimum* were less active against studied parasites than the crude extracts of the same plant. Indeed, the IC_{50} values of antitrypanosomal activities of these extracts were lower than $15 \mu\text{g/mL}$. This activity also depended on vegetative stages and on the plant part extracted. Whatever the vegetative stages, the ethanol extracts of the stems were less active ($IC_{50}=13.99 \pm 0.70 \mu\text{g/mL}$ in pre-flowering and $IC_{50}=6.52 \pm 0.52 \mu\text{g/mL}$ in full flowering) than leaves extracts ($IC_{50}=6.18 \pm 0.28 \mu\text{g/mL}$ in pre-flowering and $IC_{50}=1.66 \pm 0.48 \mu\text{g/mL}$ in full flowering) which were less active than seeds ones ($IC_{50}=1.29 \pm 0.42 \mu\text{g/mL}$ in full flowering). We also observed that the extracts in full flowering were more active against *Trypanosoma brucei brucei* than in pre-flowering. Leaves and seeds ethanol extracts showed good antitrypanosomal activities with IC_{50} values lower than $2 \mu\text{g/mL}$.

Concerning the antiplasmodial activity against the chloroquine-sensitive strain of *Plasmodium falciparum* (3D7), we observed that it depended on vegetative stages and on the plant part extracted but studied essential oils and crude extracts obtained in pre and full

flowering showed low activities with $IC_{50} > 40 \mu\text{g/mL}$. These results were close to those observed with the leaf methanol extracts of the plant from South India ($IC_{50}=32 \mu\text{g/mL}$) on the same strain (Kamaraj et al., 2012), but far from those observed with ethyl acetate extract of leaves from Nigeria against chloroquine/pyrimethamine resistant (K1) strain ($IC_{50}=1.8 \mu\text{g/mL}$) (Abiodun et al., 2011).

3.4. Cytotoxicity on CHO and WI38 and toxicity on *Artemia salina* Leach. larvae

The cytotoxicity tests against CHO and WI38 cells showed that all tested oils and components had a low cytotoxicity ($IC_{50} > 50 \mu\text{g/mL}$) (Table 3) excepted leaves and seeds ethanol extracts obtained in full flowering stage which were cytotoxic against CHO cells ($IC_{50}=18.50 \mu\text{g/mL}$ and $10.25 \mu\text{g/mL}$ respectively) and WI38 cells ($IC_{50}=21.40 \mu\text{g/mL}$ and $14.11 \mu\text{g/mL}$ respectively). Nevertheless, they showed interesting selectivity index for antitrypanosomal activity ($SI > 10$ for leaves and seeds in full-flowering). The essential oils, leaves and stems extracts of *Ocimum gratissimum* already largely used in folk medicine and cooking seemed nontoxic in pre-flowering stage but in full flowering stage, should need further research on their toxicity.

The LC_{50} values on *Artemia salina* larvae (Table 3) vary according to vegetative stages, to the plant part and to the volatility, or not, of the extracts. Authors suggested that substances could be regarded as toxic if the LC_{50} value is inferior to $30 \mu\text{g/mL}$ (Meyer et al., 1982). In our study LC_{50} values ranged between $42 \mu\text{g/mL}$ and $284 \mu\text{g/mL}$. We thus can note that the samples may be considered as not toxic. The nontoxicity of the essential oils of this plant was previously reported (Kpadonou Kpoviessi et al., 2012).

4. Conclusions

The chemical composition of the essential oils of *Ocimum gratissimum* depends on the vegetative stage. The essential oils and non-volatile crude extracts of the plant were more active on *Trypanosoma brucei brucei* than on *Plasmodium falciparum* (3D7) and this activity varies according to the vegetative stage and the extracted plant part. The crude extracts were more active than the essential oils and their major compounds, and the most active extracts were seeds and leaves in full flowering ($IC_{50} < 2 \mu\text{g/mL}$). The essential oils contained some minor compounds as myrcene, citronellal, limonene,... with good antitrypanosomal activities which concentration seemed diluted in major non active compounds. These oils and crude extracts were nontoxic against *Artemia salina* Leach and had a low cytotoxicity against CHO and WI38 cells with the exception of leaves and seeds ethanol extracts obtained in full flowering which presented also the best antitrypanosomal activities. Nevertheless their selectivity indexes were around 10, showing an antitrypanosomal potential of *Ocimum gratissimum* and confirming the use in folk medicine and cooking. However, it should need further research on its toxicity especially on the plant in full flowering and the population sensitized about it.

Table 3
In vitro antitrypanosomal and antiplasmodial activity, cytotoxicity, toxicity against *Artemia salina* L. and selectivity index of crude extracts, essential oils from *Ocimum gratissimum* Linn and some of their major components.

Samples		Toxicity (LC ₅₀ , µg/mL) mean ± standard deviation	Cytotoxicity (IC ₅₀ , µg/mL) average ± standard deviation		Antitrypanosomal activity <i>Tbb</i> (IC ₅₀ , µg/mL) mean ± standard deviation	Antiplasmodial activity <i>Pf</i> (IC ₅₀ , µg/mL) mean ± standard deviation	Selectivity indices		
			<i>Artemia salina</i> L	CHO			WI38	WI38/ <i>Tbb</i>	3D7/ <i>Tbb</i>
Pre-flowering	I	70.40 ± 0.03 ^c	125.04 ± 4.27 ^e	162.15 ± 2.08 ^c	69.59 ± 7.33 ^g	55.06 ± 14.68 ^e	2.33	0.79	
	II	102.90 ± 0.02 ^d	195.88 ± 20.69 ^f	195.88 ± 20.69 ^f	13.99 ± 0.70 ^d	41.92 ± 22.19 ^b	14.00	3.00	
	III	56.10 ± 0.02 ^b	287.46 ± 3.76 ^g	287.46 ± 3.76 ^g	6.18 ± 0.28 ^c	45.04 ± 22.78 ^c	46.51	7.29	
Full-flowering	I	42.90 ± 0.02 ^a	125.00 ± 1.68 ^e	165.51 ± 6.81 ^e	27.23 ± 3.74 ^e	49.29 ± 12.35 ^d	6.08	1.81	
	II	134.20 ± 0.03 ^e	64.83 ± 3.23 ^d	72.07 ± 3.84 ^d	6.52 ± 0.52 ^c	76.92 ± 24.32 ^g	11.05	11.79	
	III	179.30 ± 0.02 ^f	18.50 ± 0.38 ^c	21.40 ± 1.20 ^c	1.66 ± 0.48 ^b	59.99 ± 21.96 ^f	12.89	36.13	
	IV	284.00 ± 0.02 ^g	10.25 ± 0.33 ^b	14.11 ± 1.11 ^b	1.29 ± 0.42 ^b	51.86 ± 28.87 ^d	10.94	40.21	
Pure compounds*	myrcene		> 50	> 50	2.24 ± 0.27 ^b	nd	> 22.32		
	R(+)-limonene		> 50	> 50	4.24 ± 1.27 ^{bc}	nd	> 11.79		
	citronellal		> 50	> 50	2.76 ± 1.55 ^b	nd	> 18.12		
	Nerol		> 50	> 50	> 100	nd	< 0.5		
	β-citronellol		> 50	> 50	6.45 ± 4.86 ^c	nd	> 7.75		
	β-pinene		> 50	> 50	47.37 ± 15.65 ^f	nd	> 1.06		
	6-acetoxy-p-mentha-1,8-diene		> 50	> 50	28.82 ± 2.91 ^e	nd	> 1.74		
	p-cymene		> 50	> 50	76.32 ± 13.27 ^h	nd	> 0.66		
	Positive controls	Camptothecin	13.27 ± 0.02	0.74 ± 0.09 ^a	0.44 ± 0.12 ^a	nd	nd		
		Suramine	nd	nd	nd	0.11 ± 0.02 ^a	nd		
	Chloroquine	nd	nd	nd	nd	0.02 ± 0.01 ^a			
	Artemisinin	nd	nd	nd	nd	0.01 ± 0.001 ^a			

I=Essential oil from aerial part of *O. gratissimum* collected at 7 am, II=Ethanollic Extract stems of *O. gratissimum*, III=Ethanollic Extract leaves of *O. gratissimum*, IV=Ethanollic Extract seeds of *O. gratissimum* in full-flowering, WI38=human normal fibroblast cells, CHO=Chinese Hamster Ovary cells, nd=not determined, *Tbb*=*Trypanosoma brucei*, 3D7=Chloroquine-sensitive strain of *Plasmodium falciparum*, IC₅₀=sample concentration providing 50% death of cells or parasites, LC₅₀=sample concentration providing 50% death of larvae, *Selectivity index=IC₅₀ (WI38)/IC₅₀ (*Tbb* or 3D7). Data in the same column followed by different letters (^{a,b,c,...}) are statistically different by Student's *t*-test (*P* < 0.05). Values are means ± standard deviation of three different experiments.

* Kpoviessi et al., 2014.

This is the first report about the influence of plant part and vegetative stages on the antitrypanosomal and antiplasmodial activities and on cytotoxicity of the essential oil and crude extracts of *Ocimum gratissimum* Linn from Benin.

Author agreement

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

Acknowledgment

A part of this work was supported by the CUD (Commission Universitaire pour le Développement) and CIUF (Coopération Institutionnelle Universitaire Francophone) of Belgium. One of us (Yayi E.) is very indebted to the International Foundation for Science for partial support of this work through grant no. 2559-2. The authors gratefully thank the Belgian National Fund for Scientific Research (FNRS) (T.0190.13 and 3.4533.10).

References

Abiodun, O., Gbotosho, G., Ajaiyeoba, E., Happi, T., Falade, M., Wittlin, S., Sowunmi, A., Brun, R., Oduola, A., 2011. *In vitro* antiplasmodial activity and toxicity assessment of some plants from Nigerian ethnomedicine. *Pharmaceutical Biology* 49 (1), 9–14.

Adams, R.P., 1995. *Identification of Essential Oil Components by Gas Chromatography and Mass Spectrometry*. Allured Publ. Corp., Carol Stream, IL, USA.

Adamu, M., Nwosu, C.O., Agbede, R.I.S., 2009. Anti-trypanosomal effects of aqueous extract of *Ocimum gratissimum* (Lamiaceae) leaf in rats infected with *Trypanosoma brucei brucei*. *African Journal of Traditional, Complementary and Alternative Medicines* 6 (3), 262–267.

Adebolu, T.T., Salau, A.O., 2005. Antimicrobial activity of leaf extracts of *Ocimum gratissimum* on selected diarrhoea causing bacteria in southwestern Nigeria. *African Journal of Biotechnology* 4, 682–686.

Adjadohoun, E.J., 1986. *Médecine traditionnelle et Pharmacopée: Contributions aux Etudes Ethnobotaniques et Floristiques au Togo*. A.C.C.T, Paris, pp. 160–248.

Adjadohoun, E.J., 1989. *Médecine traditionnelle et pharmacopée: contributions aux Etudes Ethnobotaniques et Floristiques en République du Bénin*. A.C.C.T, Paris, pp. 274–356.

Adjadohoun, E.J., Ake Assi, L., 1979. Contribution au recensement des plantes médicinales de Côte d'Ivoire. Centre National de Floristique, Abidjan.

AFNOR, 2000. *Recueil de Normes Françaises, Huiles Essentielles, Tome 1: Echantillonnage et méthodes d'analyse, NFT75-401*, Paris 207.

Akinyemi, K.O., Oladapo, O., Okwara, C.E., Ibe, C.C., Fasura, K.A., 2005. Screening of crude extracts of six medicinal plants used in South-west Nigerian unorthodox medicine for anti-methicillin resistant *Staphylococcus aureus* activity. *BMC Complement Alternative Medicine* 5, 6–10.

Ayedoun, M.A., 1995. (Ph.D. Thesis). Contribution à la connaissance chimique des huiles essentielles de plantes aromatiques du Bénin en vue de leur valorisation. Nationale University of Benin at Cotonou.

Bissangou, M.F., 1993. (Ph.D. Thesis). Contribution à l'étude des composés organiques volatils extraits de plantes aromatiques d'Afrique Subsaharienne. University of Montpellier, pp. 140–149.

Borges, A.R., Aires, J.R., Higinio, T.M., de, M.M., Cito, A.M., Lopes, J.A., de Figueiredo, R. C., 2012. Trypanocidal and cytotoxic activities of essential oils from medicinal plants of North-east of Brazil. *Experimental Parasitology* 132, 123–128.

Bruneton, J., 1993. *Pharmacognosie: Phytochimie, plantes médicinales*, 2ème édition Technique et Documentation-Lavoisier, Paris, pp. 387–404.

Clevenger, J.F., 1928. Apparatus for the deterioration of volatile oil. *Journal of American Pharmacy Association* 17, 346.

Danzel, A.C., 1996. *The Useful Plants of West Africa*. Crown Agents, London p. 162.

Dubey, N.K., Kishore, N., Vanna, J., Lee, S.Y., 1997. Cytotoxicity of the essential oils of *Cymbopogon citratus* and *Ocimum gratissimum*. *Indian Journal of Pharmaceutical Sciences* 59, 263–267.

Heneberg, D., Weimann, B., Jopperk, W., 1995. Mass spectrometry library search system masslib, Version 7.4 (for Ultrix), Max-Planck-Institut für Kohlenforschung, mülheim/Rhur (1994);

Heneberg, D., Weimann, B., Jopperk, W., MPI library of mass spectral data, Max-Planck-Institut für Kohlenforschung, mülheim/Rhur, 1994; Leclercq, P.A., Snijders, H.M., EUT library of EI mass spectra, Eindhoven University of Technology.

Hirumi, H., Hirumi, K., 1994. Axenic culture of African trypanosome bloodstream forms. *Parasitology Today* 10, 80–84.

- Hoet, S., Opperdoes, F., Brun, R., Adjakidjé, V., Quetin-Leclercq, J., 2004. *In vitro* antitrypanosomal activity of ethnopharmacologically selected Beninese plants. *Journal of Ethnopharmacology* 91, 37–42.
- Ilori, M., Sheteolu, A.O., Omonibgehin, E.A., Adeneye, A.A., 1996. Antidiarrhoeal activities of *Ocimum gratissimum* (Lamiaceae). *Journal of Diarrhoeal Diseases Research* 14, 283–285.
- Janine, A.L., Xisto, S.P., Orionalda, F.L.F., Jose, R.P., Pedro, H.F., Lucia, K.H.S., Aline, A.L., Maria, R.R.S., 2005. Antifungal activity from *Ocimum gratissimum* L. towards *Cryptococcus neoformans*. *Memorias do Instituto Oswaldo Cruz* 100, 55–63.
- Kamaraj, C., Kaushik, N.K., Mohanakrishnan, D., Elango, G., Bagavan, A., Zahir, A.A., Rahuman, A.A., Sahal, D., 2012. Antiplasmodial potential of medicinal plant extracts from Malaiyur and Javadhu hills of South India. *Parasitology Research* 111 (2), 703–715.
- Kaou, A.M., Mahiou-Leddet, V., Hutter, S., Ainouddine, S., Hassani, S., Yahaya, I., Azas, N., Ollivier, E., 2008. Antimalarial activity of crude extracts from nine African medicinal plants. *Journal of Ethnopharmacology* 116, 74–83.
- Kpadonou Kpoviessi, B.G.H., Kpoviessi, D.S.S., Yayi Ladekan, E., Gbaguidi, F., Yehouenou, B., Moudachirou, M., Accrombessi, G.C., 2013. Phytochemical screening, antimicrobial activities and toxicity against *Artemia salina* Leach of extracts and fractions of *Ocimum gratissimum* L. from Benin. *Journal of Chemical and Pharmaceutical Research* 5 (10), 369–376.
- Kpadonou Kpoviessi, B.G.H., Yayi Ladekan, E., Kpoviessi, D.S.S., Gbaguidi, F., Yehouenou, B., Quetin-Leclercq, J., Figuereido, G., Moudachirou, M., Accrombessi, G.C., 2012. Chemical Variation of Essential Oil Constituents of *Ocimum gratissimum* L. from Benin, and Impact on Antimicrobial Properties and Toxicity against *Artemia salina* Leach. *Chemistry & Biodiversity* 9 (1), 139–150.
- Kpoviessi, S., Bero, J., Agbani, P., Gbaguidi, F., Kpadonou-Kpoviessi, B., Sinsin, B., Accrombessi, G., Frédéric, M., Moudachirou, M., Quetin-Leclercq, J., 2014. Chemical composition, cytotoxicity and *in vitro* antitrypanosomal and antiplasmodial activity of the essential oils of four *Cymbopogon* species from Benin. *Journal of Ethnopharmacology* 151, 652–659.
- Makler, M.T., Ries, J.M., Williams, J.A., Bancroft, J.E., Piper, R.C., Gibbins, B.L., Hinrichs, D.J., 1993. Parasite lactate-dehydrogenase as an assay for *Plasmodium falciparum* drug-sensitivity. *American Journal of Tropical Medicine and Hygiene* 48, 739–741.
- Mapi, J., 1988. (Ph.D. Thesis). Contribution à l'étude ethnobotanique et analyse chimique de quelques plantes utilisées en médecine traditionnelle dans la région de Nkongsamba (Cameroun). University of Yaounde, pp. 68–80.
- Masada, Y., 1967. Analysis of essential oils by gas chromatography and mass spectrometry. Wiley, New-York, pp. 193–199.
- Matasyoh, L.G., Matasyoh, J.C., Wachira, F.N., Kinyua, M.G., Muigai, A.W.T., Mukiana, T.K., 2007. Chemical composition and antimicrobial activity of the essential oil of *Ocimum gratissimum* L. growing in Eastem Kenya. *African Journal of Biotechnology* 6, 760–765.
- Mbata, T.I., Saikia, A., 2005. Antibacterial Activity of Essential oil from *Ocimum gratissimum* on *Listeria monocytogenes*. *Internet Journal of Food Safety* 7, 15–19.
- Mclafferty, F.W., Stauffer, D.B., 1991. Mass Spectrometry Library Search System BenchTop/PBM, Version 3.0, Palisade Co, Newfeld, New-York NY, 1993; Using BenchTop/PBM the following database was searched: F.W. Mclafferty, D.B. Stauffer, the Wiley NBS Registry of Mass Spectral Data, 5th edition, J. Wiley and Son, New-York NY.
- Meyer, B.N., Ferrigni, N.R., Putnam, J.E., Jacobsen, L.B., Nichols, D.E., McLaughlin, J.L., 1982. Brine shrimp: a convenient bioassay for active plant constituents. *Planta Medica* 45, 31–34.
- Michael, A.S., Thompson, C.G., Abramovitz, M., 1956. *Artemia salina* as a test organism for a bioassay. *Science* 123 (3194), 464.
- Murebwayire, S., Frederich, M., Hannaert, V., Jonville, M.C., Duez, P., 2008. Antiplasmodial and antitrypanosomal activity of *Trichlisia saculeuxii* (Pierre) Diels. *Phytomedicine* 15, 728–733.
- Musuyu Muganza, D., Fruth, B.I., Nzunzu Lami, J., Mesia, G.K., Kambu, O.K., Tona, G. L., Cimanga Kanyanga, R., Cos, P., Maes, L., Apers, S., Pieters, L., 2012. *In vitro* antiprotozoal and cytotoxic activity of 33 ethnopharmacologically selected medicinal plants from Democratic Republic of Congo. *Journal of Ethnopharmacology* 141, 301–308.
- Nakamura, C.V., Ueda-Nakamura, T., Bando, E., Melo, A.F.N., Cortez, D.A.G., Filho, B.P. D., 1999. Antibacterial Activity of *Ocimum gratissimum* L. Essential Oil. *Memorias Instituto Oswaldo Cruz* 94 (5), 675–678 (Rio de Janeiro).
- Nguta, J.M., Mbaria, J.M., Gathumbi, P.K., Gakuya, D., Kabasa, J.D., Kiama, S.G., 2011. Ethnodiagnostic skills of the digo community for malaria: a lead to traditional bioprospecting. *Frontiers in Pharmacology* 2 (30), 1–14.
- Nibret, E., Wink, M., 2010. Trypanocidal and antileukaemic effects of the essential oils of *Hagenia abyssinica*, *Leonotis ocyimifolia*, *Moringa stenopetala*, and their main individual constituents. *Phytomedicine* 17, 911–920.
- NIST, 1994. (National Institute of Standards and Technology). PC Version of the NIST/EPA/NIH Mass Spectral Database, Version 4.5. US Department of commerce, Gaithersburg, MD.
- Oliveira, V.C.S., Daniel, M.S., Moura, D.M.S., Lopes, J.A.D., de Andrade, P.P., da Silva, N.H., Figueiredo, R.C.B.Q., 2009. Effects of essential oils from *Cymbopogon citratus* (DC) Stapf., *Lippia sidoides* Cham., and *Ocimum gratissimum* L. on growth and ultrastructure of *Leishmania chagasi* promastigotes. *Parasitology Research* 104, 1053–1059.
- Oliver B., 1960. *Medicinal Plants in Nigeria*. Nigerian College of Arts, Science and Technologie, Ibadan. pp. 138.
- Onajobi, F.D., 1986. Smooth muscle contracting lipidic soluble principles in chromatographic fractions of *Ocimum gratissimum*. *Journal of Ethnopharmacology* 18, 3–11.
- Orafidia, L.O., Adesina, S.K., Igbeneghu, O.A., Akinkunmi, E.O., Adetogun, G.E., Salau, A.O., 2006. The effect of honey and surfactant type on the antibacterial properties of the leaf essential oil of *Ocimum gratissimum* Linn against common wound-infecting organisms. *International Journal of Aromatherapy* 16, 57–62.
- Oussou, K.R., Yolou, S.F., Tue, B.I.D., Kanko, C., Boti, J.B., Ahibo, C., Casanova, J., 2010. Etude Chimique Bio-Guidée de L'huile Essentielle de *Ocimum gratissimum* (Lamiaceae). *European Journal of Scientific Research* 40 (1), 50–59.
- Prabhu, K.S., Lobo, R., Shirwaikar, A.A., Shirwaikar, A., 2009. *Ocimum gratissimum*: a Review of its Chemical, Pharmacological and Ethnomedicinal Properties. *Open Complementary Medicine Journal* 1, 1–15.
- Sadler Research Laboratories, 1986. The Sadler standard gas chromatography retention index library. Bio-Rad Laboratories, Philadelphia.
- Sandra, P., Bicchi, C., 1987. *Capillary Gas Chromatography in Essential Oil Analysis*. Hüthig, Heidelberg.
- SAS/STAT, 1990. (SAS Inst., Inc., Cary, NC 2(6). User's Guide. SAS Institute, Inc. 1990, 4th ed. SAS Institute, pp. 951–958.
- Sleet, R.B., Brendel, K., 1983. Improved methods for use in developmental toxicology. *Ecotoxicology and Environmental Safety* 7, 435–446.
- Sofowora, E.A., 1970. A study of the variations in essential oil of cultivated *Ocimum gratissimum*. *Planta Medica* 18, 173–176.
- Steel, R.G.D., Torrie, J.H., 1980. *Principles and Procedures of Statistics*, 2nd ed. McGraw-Hill, NewYork.
- Stevigny, C., Block, S., Pauw-Gillet, M.C., de Hoffmann, E., Llabres, G., Adjakidjé, V., Quetin-Leclercq, J., 2002. Cytotoxic aporphine alkaloids from *Cassipouira filiformis*. *Planta Medica* 68, 1042–1044.
- Sulistiari, D., 1999. *Ocimum gratissimum* L. In: Oyen, L.P.A., Dung, Nguyen Xuan (Eds.), *Plant Resources of South-East Asia. Essential-oils plants*, 19. Indonesia: Prosea Foundation, Bogor, pp. 140–142.
- Tchoumboungang, F., Zollo, P.H., Dagne, E., Mekonnen, Y., 2005. *In vivo* antimalarial activity of essential oils from *Cymbopogon citratus* and *Ocimum gratissimum* on mice infected with *Plasmodium berghei*. *Planta Medica* 71, 20–23.
- Terezinha, J.F., Rafael, S.F., Lidiane, Y., Jose, R.P.S., Noemia, K.I., Aneli, M.B., 2006. Antifungal Activity of Essential Oil Isolated from *Ocimum gratissimum* L. (eugenol chemotype) against Phytopathogenic Fungi. *Brazilian Archives Biology and Technology* 49, 867–871.
- Tona, G.L., Mesia, G.K., Nanga, T.H., Cimanga, R.K., Apers, S., Cos, P., Maes, L., Pieters, L., Vlietinck, A.J., 2007. *In vitro* antiprotozoal and cytotoxic activities of plant extracts from Democratic Republic of Congo. *Recent Research Developments in Plant Science* 4, 41–60.
- Trager, Jensen, J.B., 1976. Human malaria parasites in continuous culture. *Science* 193, 673–675.
- Vanhaecke, P., Persoone, G., Claus, C., Sorgeloos, P., 1981. Proposal for a Short-term toxicity test with *Artemia nauplii*. *Ecotoxicol Environment Safety* 5, 382–387.
- Yayi, E., 1998. (Ph.D. Thesis). Contribution à l'étude des huiles essentielles de plantes aromatiques du Bénin; cas des *Ocimum basilicum*, *canum* et *gratissimum* dans la perspective de leur production. University Blaise Pascal-Clermont-Ferrand II. National University of Benin at Cotonou.
- Yayi, E., Gbenou, J.D., Ahoussi, L.A., Moudachirou, M., Chalchat, J.C., 2004. *Ocimum gratissimum* L., siège de variations chimiques complexes au cours du développement. *Compte Rendu de l'Académie des Sciences* 7, 1013–1018.