Chemical Variation of Essential Oil Constituents of *Ocimum gratissimum* L. from Benin, and Impact on Antimicrobial Properties and Toxicity against *Artemia salina* LEACH

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To determine the period of harvest that optimizes the antimicrobial activities of the essential oil of *Ocimum gratissimum* L. from Benin, aerial plant parts were collected at two vegetative stages (pre- and full-flowering) and three sampling times (7 am, 1 pm, and 7 pm). Extraction by hydrodistillation yielded between 0.65 and 0.78% of essential oils. Characterization of the oils by GC-FID and GC/MS analysis revealed the presence of monoterpenes (87.26–93.81%), sesquiterpenes (5.57–11.34%), and aliphatic compounds (0.15–0.18%), with *p*-cymene (**1**; 28.08–53.82%), thymol (**2**; 3.32–29.13%), γ-terpinene (**3**; 1.11–10.91%), α-thujene (**4**; 3.37–10.77%), and β-myrcene (**5**; 4.24–8.28%) as major components. Two chemotypes were observed, *i.e.*, a *p*-cymene/thymol and a *p*-cymene chemotype, for plants harvested at 7 am for the former and at 1 pm or 7 pm for the latter, respectively. The oils were fungicidal against *Candida albicans*, with the sample from full-flowering plants collected at 7 am being the most active ($MIC = 0.06 \pm 0.00$ mg/ml). The chemical variation of the oils also influenced the antimicrobial effect against *Staphylococcus aureus*; the most active oil was obtained from plants at the pre-flowering stage collected at 7 am ($MIC = 0.24 \pm 0.01$ mg/ml). *Escherichia coli* was insensitive to the chemical variation of the oils (*MIC*s of *ca.* 0.48 ± 0.02 mg/ml for all oils). Moreover, the essential oils showed low toxicity against *Artemia salina* LEACH larvae, with LC_{50} values in the range of 43–146 μg/ml. This is the first study of the interaction between the daytime of collection and vegetative stage of the plants and the antimicrobial properties and toxicity of the essential oil of *O. gratissimum* from Benin.

Introduction. – Originating from Southeast Asia, *Ocimum gratissimum* L. is a perennial and odoriferous shrub currently present on all the continents and whose therapeutic virtues are universally recognized [1][2]. In Africa, its therapeutic use is extremely broad and varies according to the countries [3]. In Cameroun, infusions of the aerial parts are considered tonic and pectoral, and the juice of its sheets is used to relieve headaches, giddiness, cold, and cough [4]. In Ivory Coast, ophthalmias, otitises,

and dermatoses are treated with various preparations of this plant [5]. In Nigeria, *Olivier* [6] prescribed it in diarrhea treatment, whereas *Sofowora* [7] indicated it for respiratory affections and as anthelmintic. The same virtues were recognized in Rwanda. In Togo, infusions of the plant are used as antitussive, the juice of its fresh leaves as antidiarrheic and antidysenteric, and its aqueous maceration is used in the treatment of hematuries and purulent urethritis [8][9]. In Benin, the aqueous maceration of its pulp or aerial parts is used to cure dystopias, pelvic pains, dysmenorrhoeas, colics, candidoses, vomiting, hemorrhoids, and diarrhea. Decoctions of the stems are used in the treatment of hepatitis, cough, asthma, and wound infections [9][10]. The juice of the leaves is used in anginas, cephalgias, and malnutrition. The plant inflorescences are used in the composition of many foods as aromatizing ingredient [8]. It is usually sold on the markets for its condimental and medicinal properties [11].

Previous work has reported the presence in Beninese *O. gratissimum* essential oil of the three compounds *p*-cymene (**1**), thymol (**2**), and γ-terpinene (**3**), which were not dissociable because of the rapid and easy interconversion of one compound into another during the growing process of the plant, within the same plant during daytime, and after the harvest of the vegetal material. That has led to the multiplicity of chemical profiles reported for the essential oils of *O. gratissimum* from Benin.

Moreover, the antimicrobial activity of the essential oils of *O. gratissimum* was recognized in several countries such as in Brazil, Nigeria, Ivory Coast, Cameroun, Kenya, and Togo [13–22]. But, to the best of our knowledge, no previous work has reported the influence of the daytime of harvest and the vegetative stages on the chemical variation of the essential oil constituents of *O. gratissimum* from Benin, on its antimicrobial properties, and on its toxicity. To determine the best moment of harvest, which allows to obtain oils with optimized antimicrobial activities and reduced toxicity, the aim of this work was to evaluate the influence of the chemical variation over the daytime and vegetative stages of *O. gratissimum* essential oils from Benin on their antimicrobial properties and their toxicity towards *Artemia salina* LEACH.

Results and Discussion. – *Variation of the Yield and Chemical Composition of* Ocimum gratissimum *Essential Oil.* The yields of the *O. gratissimum* essential oils slightly varied, depending on the vegetative stage and the daytime of harvest of the plants (*Table 1*). These yields (0.65–0.78% in average) ranged between 0.4 and 0.8% and were comparable to those found in a previous study [12]. The pre-flowering plants gave slightly less oil (*ca*. 0.68% in average) than the full-flowering ones (*ca*. 0.74% in average). Moreover, the essential oil yield from plants collected at 1 pm was slightly lower than those from plants harvested at 7 am and 7 pm, probably due to a volatilization under the effect of sunlight at that moment. Statistical analysis showed that the oil yield from plants collected at the pre-flowering stage at 1 pm was significantly lower than those from plants at the full-flowering stage harvested at 7 am and 7 pm (*Table 1*).

Analysis by GC-FID and GC/MS of the essential oils of *O. gratissimum* from the South of Benin, collected at various moments of the day and two vegetative stages, provided data on the chemical compositions (*Table 2*) which, qualitatively, confirmed the results of previous studies [12].

Table 1. *Influence of the Vegetative Stage and Sampling Time on the Yields* [% (*w*/*w*)] *of* Ocimum gratissimum *Essential Oil* ($n=3$)

Daytime of harvest	Pre-flowering	Full-flowering
7 am	0.71 ± 0.01 (ab)[a])	0.78 ± 0.01 (b)
1 pm	0.65 ± 0.01 (a)	0.70 ± 0.02 (ab)
7 pm	0.69 ± 0.02 (ab)	0.75 ± 0.01 (b)

[a]) Means with different letters in parentheses within the same column differ significantly by the *Newman–Keuls* test ($p \leq 0.05$).

All analyzed samples contained monoterpenes (87.26–93.81%), sesquiterpenes (5.57–11.34%), and aliphatic compounds (0.15–0.18%), among which hydrocarbon compounds were more abundant than oxidized ones. The aliphatic compounds constituted the smallest chemical group. Their percentages varied only very slightly, according to the daytime of harvest and the vegetative stage. The monoterpenes made up the major group of chemical compounds. Their percentages were very similar (*ca.* 90%) in the oils of plants at the pre-flowering stage, but slightly lower (*ca.* 87%) in the oil of plants at the full-flowering stage collected at 1 pm. Monoterpene hydrocarbons were slightly less abundant in plants at the pre-flowering stage than in full-flowering plants. Lower in plants collected in the morning at 7 am (55.87 and 56.72% in pre- and full-flowering plants, resp.), their percentages rose until the evening to reach 76.15% in pre-flowering and 86.48% in full-flowering plants harvested at 7 pm. This trend was reversed for the content of oxygenated monoterpenes, which were more abundant in the morning (32.96 and 35.43% at the pre- and full-flowering stage, resp.) and decreased until the evening at 7 pm (13.25 and 7.33% at the pre- and full-flowering stage, resp.). Hence, the total percentage of monoterpenes in the essential oils was more or less constant. The five major compounds of all the analyzed samples belonged to the chemical group of the monoterpenes (*Table 2* and *Fig. 1*). They were, in the order of decreasing contents, *p*-cymene (**1**; 28.08–53.82%), thymol (**2**; 3.32–29.13%), γ-terpinene (**3**; 1.11–10.91%), α-thujene (**4**; 3.37–10.77%), and β-myrcene (**5**; 4.24–8.28%). The contents of each of these compounds varied according to the vegetative stage and the daytime of harvest, but their sum remained almost constant, especially in the pre-flowering plants. The percentages of **1**, **4**, and **5** rose from the morning until the evening for both studied stages, while the percentages of **2** and **3** decreased during the day. This might be explained by the rapid and easy interconversion of one of the compounds into another under the effect of the sun [12].

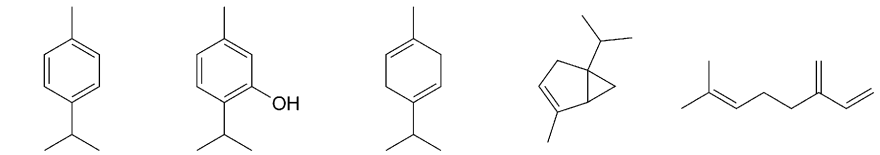

Fig. 1. *Chemical structure of the major essential oil constituents isolated from the aerial parts of* Ocimum gratissimum

Table 2. *Chemical Composition of the Essential Oils Obtained from the Aerial Parts of* Ocimum gratissimum *Harvested at Two Flowering Stages and Three Daytimes*

Compounds	*KI*[a])	Content [%]					
		Pre-flowering			Full-flowering		
		7 am	1 pm	7 pm	7 am	1 pm	7 pm
Aliphatic compounds		*0.18*	*0.18*	*0.15*	*0.16*	*0.18*	*0.16*
Octan-3-one	987	0.18	0.18	0.15	0.16	0.18	0.16
Monoterpenes		*88.83*	*90.12*	*89.4*	*92.15*	*87.26*	*93.81*
Monoterpene hydrocarbons		*55.87*	*70.43*	*76.15*	*56.72*	*71.36*	*86.48*
***α*-Thujene (4)**	**927**	**3.37**	**6.42**	**6.52**	**3.86**	**4.05**	**10.77**
α-Pinene	933	0.88	1.58	1.6	0.95	1.13	2.59
Camphene	948	0.11	0.19	0.19	0.12	0.15	0.3
Sabinene	973	0.93	1.25	1.43	0.89	0.11	2.1
β-Pinene	976	0.52	0.6	0.7	0.41	0.52	0.85
***β*-Myrcene (5)**	**991**	**4.24**	**6.01**	**6.52**	**4.53**	**5.69**	**8.28**
α-Phellandrene	1003	0.15	0.21	0.17	0.22	0.11	0.12
δ-Car-3-ene	1011	0.19	0.29	0.3	0.22	0.26	0.36
α-Terpinene	1017	1.93	2.37	2.13	2.35	2.41	1.62
***p*-Cymene (1)**	**1027**	**31.53**	**39.55**	**46.55**	**28.08**	**47.96**	**53.82**
Limonene	1029	1.43	1.34	1.64	1.53	1.53	1.72
β-Phellandrene	1031	0.34	0.42	0.46	0.44	0.44	0.53
(*Z*)-β-Ocimene	1038	0.27	0.26	0.24	0.13	0.13	0.12
(*E*)-β-Ocimene	1048	0.13	0.11	0.1	0.05	0.05	0.03
***γ*-Terpinene (3)**	**1069**	**7.69**	**7.53**	**5.47**	**10.91**	**4.07**	**1.11**
Terpinolene	1088	0.16	0.15	0.12	0.16	0.28	0.05
p-Cymenene	1090	1.88	2.04	1.93	1.76	2.41	2
p-Mentha-1,3,8-triene	1111	0.12	0.11	0.08	0.11	0.06	0.11
Oxygenated monoterpenes		*32.96*	*19.69*	*13.25*	*35.43*	*15.9*	*7.33*
1,8-Cineole	1034	0.21	–	0.15	0.09	0.15	0.04
cis-Sabinene hydrate	1069	1.33	1.07	1.25	1.22	1.34	1.54
Linalol	1096	0.33	0.25	0.2	0.16	0.23	0.13
trans-Sabinene hydrate	1098	0.36	0.29	0.29	0.33	0.59	0.28
trans-Thujone	1118	0.25	0.21	0.2	0.22	0,32	0.2
Citronellal	1154	0.15	0.06	0.05	0.06	0.07	0.05
Umbellulone	1171	0.09	0.32	0.22	0.07	0.36	0.18
Borneol	1172	0.44	0.27	0.24	0.69	0.31	0.22
Terpinen-4-ol	1183	2.1	1.38	0.9	1.71	1.52	0.51
p-Cymen-8-ol	1189	0.43	0.2	0.13	0.25	0.22	0.1
α-Terpineol	1192	0.3	0.19	0.14	0.17	0.19	0.08
Methyl thymol ether	1238	0.43	0.35	0.32	0.48	0.85	0.47
Estragol	1274	0.14	0.08	0.05	0.09	0.08	0.02
p-Cymen-7-ol	1288	0.14	0.07	0.05	0.09	–	0.04
Thymol (2)	**1311**	**25.43**	**14.5**	**8.72**	**29.13**	**9.3**	**3.32**
Carvacrol	1315	0.83	0.45	0.34	0.67	0.37	0.15
Sesquiterpenes		*10.46*	*8.89*	*9.05*	*7.22*	*11.34*	*5.57*
Sesquiterpene hydrocarbons		*9.25*	*7.86*	*8.17*	*6.33*	*10.45*	*5.08*
α-Copaene	1380	0.12	0.1	0.11	0.06	0.13	0.08
β-Elemene	1398	0.29	0.21	0.23	0.15	0.11	0.16
β-Caryophyllene	1429	2.93	2.48	2.76	2.43	3.82	2.00
α-*trans*-Bergamotene	1433	0.34	0.26	0.3	0.15	0.27	0.09

Table 2 (cont.)

Compounds	*KI*[a])	Content [%]					
		Pre-flowering			Full-flowering		
		7 am	1 pm	7 pm	7 am	1 pm	7 pm
α-Humulene	1463	0.43	0.37	0.41	0.31	0.57	0.25
Germacrene D	1483	0.2	0.15	0.19	0.41	0.17	0.17
γ-Selinene	1485	0.19	0.17	0.17	–	0.2	0.07
β-Selinene	1491	3.41	2.95	2.88	2.01	3.75	1.63
α-Selinene	1494	1.06	0.92	0.89	0.63	1.16	0.49
7-*epi*-α-Selinene	1528	0.28	0.25	0.23	0.18	0,27	0.14
Oxygenated sesquiterpenes		*1.21*	*1.03*	*0.88*	*0.89*	*0.89*	*0.49*
Caryophyllene oxyde	1596	1.11	0.94	0.81	0.82	0.81	0.45
1,2-Epoxydehumulene	1622	0.1	0.09	0.07	0.07	0.08	0.04
Total		*99.47*	*99.19*	*98.6*	*99.53*	*98.78*	*99.54*

[a]) *KI: Kovats* index determined on a *DB-5* GC column.

Compounds **1** and **2** were the major constituents in the samples harvested at 7 am (*Fig. 2*). In the other samples, only **1** was predominant, while the concentration of **2** decreased. Hence, essential oils collected at different times of the day showed different quantitative compositions, some being closer to those of essential oils isolated from *O. gratissimum* of North Benin [11] and Cameroun [23–27]. This may have an effect on their antimicrobial activities.

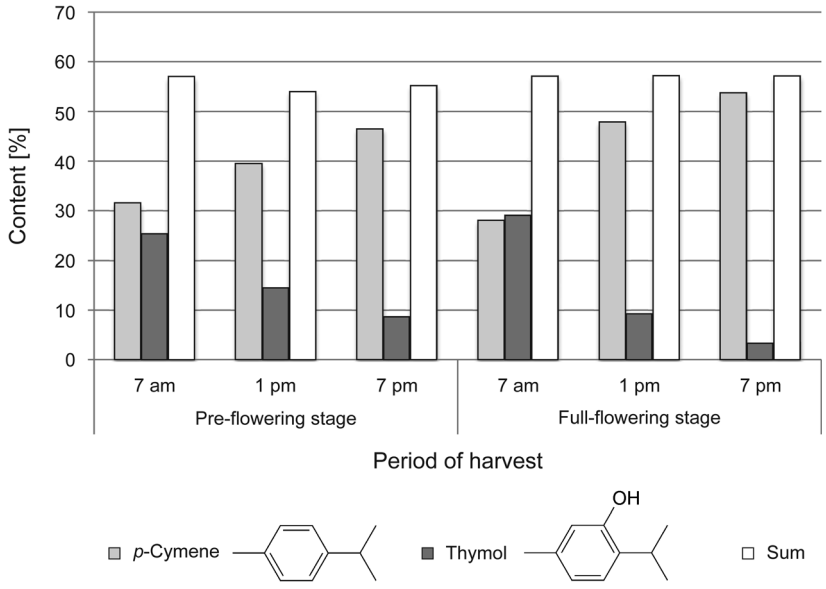

Fig. 2. *Variation of the percentages of* p-*cymene* (**1**), *thymol* (**2**), *and their sum in the essential oils isolated from the aerial parts of* Ocimum gratissimum *harvested at two flowering stages and three daytimes*

Vasconcelos et al. [28] reported that the chemical constituents of the essential oil of *O. gratissimum* from Brazil varied according to the daytime and the sun intensity. The major compounds that have been identified were eugenol and 1,8-cineole. They observed that the percentage of eugenol increased gradually at the beginning of the day to reach a maximum at noon (98%), when the sun was at the apex, before decreasing down to 11.4% at 5 pm. The percentage of 1,8-cineole, very high in the morning at 7 am (52.1%), decreased with the increasing intensity of the sun to reach its minimum at noon (less than 0.5%) and then increased again as the sun disappeared to reach its maximum at 5 pm (75%). In our study, we observed an increase during the daytime of the content of **1**, while the contrary was observed for the content of **2**. The sum of their percentages, however, remained constant (*ca.* 56%), independent of the daytime or the vegetative stage (*Fig. 2*). This observation suggests an interconversion [11] between these two components, the structures of which differed only by an OH group (*Fig. 1*). Conversion of **1** into **2** in *Thymus vulgaris* L. has previously been shown [29].

In addition, the sesquiterpene content also varied slightly according to the studied parameters. The highest percentage (11.34%) was obtained for the oils of plants at the full-flowering stage harvested at 1 pm.

Impact of the Chemical Variation of the Ocimum gratissimum *Essential Oils on Their Antimicrobial Activity.* The minimum inhibitory concentrations (*MIC*s) of the six essential oil samples were determined against a fungus (*Candida albicans* ATCC) and two bacteria (*Escherichia coli* ATCC and *Staphylococcus aureus* ATCC; *Table 3*). Nystatin and doxycycline were used as positive controls for *C. albicans* and for *E. coli* and *S. aureus*, respectively. The results showed that all oil samples were more active against the fungus than the bacteria. The essential oils of full-flowering plants were more active on *C. albicans* than those of pre-flowering plants. The oils of the plants collected at 7 am and 7 pm were more active than those of the plants harvested at 1 pm. The most active oil against *C. albicans* was obtained from full-flowering plants collected at 7 am, with a *MIC* value of 0.06 mg/ml. Hence, to treat affections due to this germ, as stomatitis, vulvo-vaginitis, urethritis, *etc.*, it is recommended to use the essential oil of plants at the full-flowering stage collected very early in the morning. All our samples were much more active against *C. albicans* than the *O. gratissimum* oil from East Kenya, containing eugenol (68.8%) as major compound (*MIC* = 50 mg/ml) [16].

The growth inhibition of *E. coli* by the oils is influenced neither by the vegetative stage of the plants, nor by the daytime of harvest (*MIC*s of *ca.* 0.47 mg/ml). The oils were more active against *E. coli* than those obtained from plants grown in Kenya (*MIC* = 107 mg/ml) [16], but less active than the oil from Ivory Coast (*MIC* = 0.016 mg/ml) [30]. Against *S. aureus*, the oil of the pre-flowering plants collected at 7 am (*MIC* = 0.24 ± 0.01 mg/ml) was more active than those of plants collected at 1 pm and 7 pm (*MIC*s of 0.49 ± 0.04 and 0.47 ± 0.02 mg/ml, resp.). The oils from full-flowering plants collected at 7 am and 7 pm (*MIC*s of 0.48 ± 0.01 and 0.47 ± 0.01 mg/ml, resp.) were more active than that of plants collected at 1 pm, which had a *MIC* value twice as high (0.95 ± 0.03 mg/ml). To sum up, pre-flowering plants collected at 7 am gave the most active oil against *S. aureus.* This Beninese oil (*MIC* = 0.24 ± 0.01 mg/ml) was more active than the *O. gratissimum* oil tested against the same germ by *Nakamura et al.* (*MIC* = 0.75 mg/ml) [13]. To treat affections caused by *S. aureus*, as furuncles, abscesses, intestinal ailments of food origin, *etc.*, it is thus recommended to use the

Table 3. *Minimal Inhibitory Concentration* (*MIC*), *Minimal Bactericidal* (*MBC*) *or Minimal Fungicidal Concentration* (*MFC*), *and Ratio* MBC/MIC *or* MFC/MIC *of the Essential Oils Obtained from the Aerial Parts of* Ocimum gratissimum *Harvested at Two Flowering Stages and Three Daytimes* ($n=3$)

Microorganism		Pre-flowering			Full-flowering			Positive control
		7 am	1 pm	7 pm	7 am	1 pm	7 pm	
C. albicans ATCC	*MIC* [mg/ml]	0.12 ± 0.00	0.25 ± 0.02	0.12 ± 0.00	0.06 ± 0.00	0.12 ± 0.00	0.12 ± 0.00	6.24 ± 0.01 [a])
	MFC [mg/ml]	0.47 ± 0.02	0.49 ± 0.04	0.47 ± 0.02	0.24 ± 0.01	0.47 ± 0.02	0.47 ± 0.01	6.24 ± 0.00 [a])
	MFC/*MIC*	3.92	1.96	3.92	4.00	3.92	3.92	1.00 [a])
E. coli ATCC	*MIC* [mg/ml]	0.47 ± 0.02	0.49 ± 0.04	0.47 ± 0.02	0.48 ± 0.01	0.47 ± 0.02	0.47 ± 0.01	0.77 ± 0.02 [b])
	MBC [mg/ml]	> 3.80	> 3.92	> 3.80	> 3.87	> 3.80	> 3.80	0.77 ± 0.01 [b])
	MBC/*MIC*	> 8.09	> 8	> 8.09	> 8.06	> 8.09	> 8.09	1.00 [b])
S. aureus ATCC	*MIC* [mg/ml]	0.24 ± 0.01	0.49 ± 0.04	0.47 ± 0.02	0.48 ± 0.01	0.95 ± 0.03	0.47 ± 0.01	6.24 ± 0.01 [b])
	MBC [mg/ml]	0.95 ± 0,03	1.96 ± 0.15	3.80 ± 0.12	0.97 ± 0.03	1.90 ± 0.06	3.80 ± 0.08	0.77 ± 0.02 [b])
	MBC/*MIC*	3.96	4.00	8.09	2.02	2.00	8.09	0.12 [b])

[a]) Nystatin was used as positive control and the *MIC* and *MFC* values are given in μg/ml. [b]) Doxycycline was used as positive control and the *MIC* and *MBC* values are given in μg/ml.

essential oil of the pre-flowering plants collected very early in the morning. Moreover, an early-morning harvest seems to be indicated for a higher inhibition of *C. albicans* and *S. aureus.*

The minimal bactericidal or minimal fungicidal concentrations (*MBC* or *MFC*, resp.) of the various samples against the three germs (*Table 3*) confirmed the observations made on the *MIC* values. *C. albicans* remained the most sensitive to the oil of the studied germs, with a low *MFC* (0.24 ± 0.01 mg/ml) obtained for the oil from plants at the full-flowering stage harvested at 7 am. *E. coli* was the less sensitive germ, with *MBC*s higher than the highest oil concentrations used in the tests. Against *S. aureus*, the lowest *MBC* (0.95 ± 0.03 mg/ml) was also obtained for the oil from pre-flowering plants collected at 7 am. To better appreciate the antimicrobial properties of the tested samples, the ratio *MBC*/*MIC* (or *MFC*/*MIC*) [30] was calculated for each oil (*Table 3*), to indicate a bactericidal (*MBC*/*MIC* or *MFC*/*MIC* ≤ 4) or bacteriostatic (*MBC*/*MIC* or *MFC*/*MIC* > 4) activity [30]. All the essential oils may be considered as fungicidal against *C. albicans* and bacteriostatic against *E. coli*. Against *S. aureus*, the daytime of harvest was of upmost importance for a bactericidal or bacteriostatic action. Indeed, bactericidal effects were obtained against this bacterium for the oils of plants collected at 7 am and 1 pm, whereas bacteriostatic effects were observed for the oils of plants collected at 7 pm.

Toxicity towards Artemia salina *Larvae.* To assess the toxicity towards *A. salina* larvae, LC_{50} values for the various essential oils were determined (*Table 4*). The toxicity test used was a preliminary method of estimation of the *in vitro* toxicity of the oils, and an extrapolation of the results to the toxicity against human cells in culture seems difficult. Nevertheless, correlations have been reported between the shrimp larvae toxicity and the cytotoxicity against 9PS (murine lymphocytic leukemia) and 9KB (human nasopharyngeal carcinoma) [31], A-549 (lung carcinoma), and HT-29 (colon carcinoma) cells [32]. Moreover, *Meyer et al.* [33] suggested that compounds could be regarded as toxic, if the LC_{50} value is inferior to 30 μg/ml. In our study, the LC_{50} values ranged between 43 and 146 μg/ml. We thus hypothesize that the samples may be considered as not toxic.

Table 4. *Toxicity* (LC_{50} [μg/ml]) *of the Essential Oils Obtained from the Aerial Parts of* Ocimum gratissimum *Harvested at Two Flowering Stages and Three Daytimes towards* Artemia salina ($n = 3$)

Daytime of harvest	Pre-flowering	Full-flowering
7 am	70.4 ± 0.03	42.9 ± 0.02
1 pm	145.0 ± 0.06	146.0 ± 0.06
7 pm	70.4 ± 0.03	120.0 ± 0.04

Conclusions. – The chemical composition of the essential oil of *O. gratissimum* varies according to the daytime of harvest and the vegetative stage of the plants. The essential oil with the highest bactericidal activity against *S. aureus* was obtained from pre-flowering plants collected at 7 am. Against *C. albicans*, all oils showed a fungicidal action, but the most active oil was obtained from full-flowering plants harvested at 7 am. *E. coli* was less sensitive to the oil. Indeed, a similar bacteriostatic action, which

was not dependent on the daytime of harvest and the vegetative stage of the plants, was observed for all oils. This is the first report about the influence of the time of harvest and the vegetative stage of the plants on the chemical composition of the essential oil of *O. gratissimum* from Benin and their impact on the antimicrobial properties of this oil. It was also shown that the oil may be considered as nontoxic against *Artemia salina* larvae.

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Experimental Part

Plant Material. The aerial parts of *Ocimum gratissimum* L. were collected during the dry season in Abomey-Calavi at Mariagleta (south of the Republic of Benin) at two vegetative stages (pre- and full-flowering) and three daytimes (7 am, 1 pm, and 7 pm) and identified by the National Herbarium of the University of Abomey-Calavi (Benin). Vouchers specimens (AA6381/HNB) have been deposited with the National Herbarium of the University.

Essential Oil Extraction. The fresh aerial parts of *O. gratissimum* were subjected to hydrodistillation during 2 h in an improved *Clevenger*-type apparatus [34][35]. The oils were preserved in a dark, cold room until GC-FID and GC/MS analyses and biological tests. The oil yields were calculated as percentages (*m*/*m*) relative to the fresh plant material.

GC-FID Analysis. The analysis of the essential oils was performed by GC-FID and GC/MS [36], and relative retention times (t_R) on three GC columns were determined [37]. The GC-FID analyses [36] were first carried out with a *DELS/IGC 121C* apparatus equipped with a split/splitless injector, a flame ionization detector (FID), and a *CP WAX 52 CB* cap. column (25 m × 0.3 mm i.d., film thickness 0.25 μm). The oven temp. was programmed isothermal at 50° for 5 min and then increasing from 50 to 220° at 2°/min; injector temp., 240°; detector temp., 250°; carrier gas, N_2 (1 ml/min); combustible gas, H_2 (30 ml/min); oxidizing gas, air (300 ml/min); injection volume, 0.5 μl.

Then, the compound separation was also achieved with a *Heweltt Packard 5890* apparatus equipped with FID, a split/splitless injector, and two glass cap. columns (30 m × 0.25 mm i.d.), one coated with *DB 5* (film thickness 0.25 μm) and the other with *Supelcowax* (film thickness 0.20 μm). The analytical conditions were identical to those described above.

GC/MS Analysis. The oils were analyzed on a *Hewlett Packard 6890* gas chromatograph coupled to a *Hewlett-Packard 5873* mass selective detector and equipped with an *HP5* column (30 m × 0.25 mm i.d., film thickness 0.25 μm).

The oven temp. was programmed linearly rising from 50 to 300° at 5°/min and then isothermal at 300° for 5 min; injector temp., 250°; detector temp., 320°; ion-source temp., 230°; carrier gas, He (1 ml/min). The samples were injected in split mode (1/10), and the electron-impact mass spectra (EI-MS; 70 eV; electron multiplier, 2200 V) were acquired over the *m*/*z* range 33–450.

The oil constituents were identified by matching their mass spectra and retention indices (*RI*) with reference libraries [37–43].

Microbial Strains. The *in vitro* antimicrobial activity of the essential oils of *O. gratissimum* was tested against laboratory control strains from the *American Type Culture Collection*, *viz*., the *Gram*-positive strain *Staphylococcus aureus* ATCC 25923, the *Gram*-negative strain *Escherichia coli* ATCC 25923, and the fungal strain *Candida albicans* ATCC 10231. All microorganisms were maintained at −20° under appropriate conditions and regenerated twice before use.

Broth Microdilution Assay. The minimal inhibitory concentration (*MIC*) of the essential oils of *O. gratissimum* was determined using the broth microdilution method in 96-well microtiter plates [44]. Inocula of the bacterial strains were prepared from overnight broth cultures, and the suspensions were adjusted to 0.5 *McFarland* standard turbidity. An aq. soln. (100 g/l) of DMSO (*Sigma–Aldrich*, St. Louis, USA) was used to dissolve and dilute the oil samples to the highest concentration to be tested (10 mg/

ml). A twofold serial dilution of the oils was prepared in each well. The final concentrations of the oils ranged from 0.005 to 10 mg/ml. The final microorganism concentration in each well was adjusted to 10^9 colony-forming units (CFU)/ml for bacteria and 10^7 spores/ml for fungal strains. Nystatin and doxycycline, in serial dilutions of 50–0.02 μg/ml, were used as positive controls for the fungus and the bacteria, resp., and the solvent was used as negative control. Microbial growth was observed by adding 10 μl of resazurin [45] indicator soln. (prepared by dissolving a 270 mg tablet in 40 ml of sterile, dist. H_2O) to the microtiter-plate wells. The plates (prepared in triplicate) were wrapped loosely with cling film (to ensure that microorganisms did not become dehydrated) and then placed in an incubator at 37° for 24 h for bacteria or at 28° for 48 h for the fungal strain. The color change was then assessed visually. A color change from purple to pink or colorless was recorded as positive for bacterial growth. The lowest concentration at which no color change occurred was taken as the *MIC* value.

The minimal bactericidal (*MBC*) or minimal fungicidal (*MFC*) concentrations were determined by spreading the content of each microtiter-plate well (50 μl) in which no color change occurred on sterile nutrient agar plates (prepared according to the manufacturer's instructions) set in *Petri* dishes [44]. These plates, after standing at 4° for 2 h to allow dispersal, were incubated at 37° for 24 h for bacteria or at 28° for 48 h for the fungal strain. The *MBC* or *MFC* was the lowest concentration of essential oil at which 99.9% of the inoculated microorganisms were killed. The tests were carried out in triplicate.

Toxicity Test against Artemia salina. The toxicity test against *A. salina* was performed according to the method of *Michael et al.* [46], as summarized by *Vanhaecke et al.* [47] and by *Sleet and Brendel* [48]. The eggs of *A. salina* were incubated in sea water until hatching of young larvae (48 h). Then, series of increasing concentrations of solns. of the oils were prepared. A defined number of larvae (16) were introduced into each soln. All test solns. and the control soln. (containing no active substance) were left under stirring for 24 h. The number of dead larvae in each soln., counted under a microscope, was used to evaluate the toxicity of the soln. In the case where there was death in the control medium, the data was corrected by *Abbott*'s equation [49]:

$$\text{Dead larvae } [\%] = (n_{\text{test}} - n_{\text{control}})/n_{\text{control}} \times 100 \quad (1)$$

where n_{test} and n_{control} are the numbers of dead larvae in the test and control solns., resp. The LC_{50} values were determined by linear regression after the logarithmic transformation of the dose–response data [50]. The tests were carried out in triplicate.

Statistical Analysis. All data were expressed as mean ± standard deviation of triplicate measurements. The confidence limit was set at $P < 0.05$. Standard deviations did not exceed 5% for the majority of the values obtained. The data were analyzed by ANOVA (analysis of variance) with the software package Statistical Analysis Systems (*SAS*) [51]. When a significant difference was observed at the level of 5%, the test of *Newman–Keuls* was used to separate the averages [52].

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