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Phytochemical screening, antimicrobial activities and toxicity against *Artemia salina* Leach of extracts and fractions of *Ocimum gratissimum Linn* from Benin

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ABSTRACT

The antimicrobial and toxicity against Artemia salina Leach of extracts from different parts (stems, leaves and seeds) of Ocimum gratissimum collected at two vegetative stages were analyzed. In addition to essential oils that contain volatile compounds and which antimicrobial properties we studied in a previous work [12], the plant contains less or non-volatile compounds that could explain in part, its properties. The crude ethanol extract of different parts (leaves, stems and seeds in pre- and full flowering) showed yields between 1.97% and 4.81%. Leaves had given more extract than seeds and the lower yield was obtained with the stems. Antimicrobial tests and toxicity against A. salina Leach helped to identify the crude extract of leaves and stems in full flowering as the most active respectively against C. albicans ATCC, and E. coli ATCC and S. aureus ATCC with a good selectivity. Phytochemical screening of different samples revealed the presence of polyphenolic compounds (gallic and catechic tannins, flavonoids, anthocyanes and leucoanthocyanes), quinone derivatives, triterpenoids, steroids, mucilage, coumarins, reducing compounds and essential oils. The liquid-liquid fractionation of the active crude extracts with different solvents of increasing polarity showed ethyl acetate fraction as the most active. This fraction needs to be further investigated by isolation and identification of pure bioactive compounds by bio-guided fractionation. This is the first report of interaction between plant parts, vegetative stages, antimicrobial properties and toxicity of the non-volatile fractions of O. gratissimum Linn from Benin.

Key words: *Ocimum gratissimum* Linn, non-volatile fraction, antimicrobial activity, selectivity index, bio-guided fractionation.

INTRODUCTION

Currently present on every continents, *Ocimum gratissimum* Linn is a perennial and odoriferous shrub from South-east of Asia and whose therapeutic virtues are universally recognized [1][2]. In Africa, its therapeutic potential is extremely broad and varies according to the countries [3]. In Cameroun, plant's infusions are considered tonic and pectoral, juice of its sheets are 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 B.* [6] prescribed it in the diarrhoeas treatment whereas Sofowora [7] indicated it for the respiratory affections and as anthelminthic. The same virtue was recognized in Rwanda. In Togo, plant's infusion is antitussive; juice of its fresh leaves is

antidiarrheic and antidysenteric; its aqueous maceration is used in hematuries and purulent urethritis treatment [8][9]. In Benin, the aqueous maceration of its pulp or aerial parts is used in dystopias, pelvic pains, digestive desorders, dysmenorrhoeas, colics, candidoses, vomiting, haemorrhoids and diarrhoea. Decoction of stems is used in the treatment of hepatitis, cough, asthma and wounds 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 [8].

It is also usually sold on the markets for its condimental and medicinal properties [11].
We studied previously the influence of the chemical variation over daytime and vegetative stages of *O. gratissimum* Linn essential oils from Benin on their antimicrobial properties and on their toxicity against *Artemia salina* Leach[12].

Moreover, the antimicrobial activities of non-volatile extracts of *O. gratissimum* Linn was known in several countries such as India, Brazil, Nigeria, Ivory Coast, Cameroun, Kenya, Togo... [13-22]. But to our knowledge, no previous work was published on the phytochemical screening, the antimicrobial activities, the toxicity against *A. salina* Leach and the fractionation of *O. gratissimum* Linn non-volatile extracts from Benin.

In order to identify, for future bio-guided fractionation, the active non-volatile extract which possesses the best antimicrobial activities with reduced toxicity, we decided to evaluate the influence of the plant part and the vegetative stages of *O. gratissimum* Linn plant from Benin on their antimicrobial properties, the plant secondary metabolites and its toxicity against *A. salina* Leach.

EXPERIMENTAL SECTION

Materials

Ocimum gratissimum Linn plant was collected (dry season) in Abomey-Calavi at Mariagleta (south of the Republic of Benin) at various vegetative stages (pre and full flowering) and identified by the National Herbarium of the University of Abomey-Calavi (Benin). Vouchers specimens (n°AA6381/HNB) have been deposited at the National Herbarium of the University. 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 were used. Chloroform, hexane, n-butanol, ethanol and ethyl acetate of HPLC grade were purchased from Fisher Fisher Scientific (Tournai, Belgium). Dimethyl sulfoxide (DMSO) were obtained from ALDRICH (St. Louis, USA), Fehling's solution (A and B), sulphuric acid (H_2SO_4), ammoniac, chloroform, ferric chloride, Shinoda, Mayer and Draggendorff's reagents, glacial acetic acid were come from Extrasynthèse (Genay, France). The eggs of *Artemia salina* Leach obtained from JBL society (JBL Gmbh&Co.KG, Germany) were used for toxicity essay.

Extraction and fractionation procedure

Seeds, leaves and stems of *O. 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. After evaporation under reduced pressure, the ethanol extracts were dried in an oven at 45 ° C to remove traces of solvent. The ethanol extracts were then dissolved in a mixture of water-ethanol (50-50) and successively extracted with hexane, chloroform, ethyl acetate and of n-butanol at room temperature, using a funnel. Each extraction was triplicated. The extracts were dried in vacuum under reduced pressure and traces of solvent were removed in an oven at 45 ° C. Each dried extract and fraction was kept in a freezer before biological tests.

Phytochemical screening

The phytochemical screening of the extracts was performed according to the standard procedures: Mayer's and dragendorff's tests for alkaloids, Fehling's test for free reducing sugers, Fehling's test for glycosides, Liebermann-burchard's test for triterpenoids, Liebermann-burchard's test for steroids, frothy test for saponins, Shinoda's and sodium hydroxide tests for flavonoids, ferric chloride test for tannins, Guignard's test for free cyanogenetics derived and Borntrager's test for free anthraquinones [23-28].

Antimicrobial activity

Microbial Strains

The *in vitro* antimicrobial activity of the crude extracts and fractions of *O. gratissimum* was tested on laboratory control strains from the American Type Culture Collection, viz., the Gram-positive strain *S. aureus* ATCC 25923, the Gram-negative strain *E. coli* ATCC 25923, and the fungal strain *C. albicans* ATCC 10231. All microorganisms were maintained at -20° C under appropriate conditions and regenerated twice before use.

Broth Microdilution Assay

The minimal inhibitory concentration (MIC) of the crud extracts and fractions of *O. gratissimum* was determined using the broth microdilution method in 96-well microtiter plates [29, 30]. Inocula of the bacterial strains were prepared from overnight broth cultures, and the suspensions were adjusted to 0.5 McFarland standard turbidity. An aqueous solution (100 g/L) of DMSO was used to dissolve and dilute the samples to the highest concentration to be tested (10 mg/ mL). A twofold serial dilution of the extracts was prepared in each well. The final concentrations of the sample 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 mg/mL, were used as positive controls for the fungus and the bacteria, respectively, and the solvent was used as negative control. Microbial growth was observed by adding 10 mL of resazurin [31] solution (prepared by dissolving a 270 mg tablet in 40 ml of sterile, distilled 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°C for 24 h for bacteria or at 28°C 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 mL) in which no color change occurred on sterile nutrient agar plates (prepared according to the manufacturer's instructions) set in Petri dishes [29].

These plates, after standing at 4°C for 2 h to allow dispersal, were incubated at 37°C for 24 h for bacteria or at 28°C for 48 h for the fungal strain. The MBC or MFC was the lowest concentration of extract 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. [32], as summarized by Vanhaecke et al. [33] and by Sleet and Brendel [34]. The eggs of *A. salina* were incubated in sea water until hatching of young larvae (48 h). Then, series of increasing concentrations of solutions of the samples were prepared in DMSO (dimethylsufoxide)/seawater. A defined number of larvae (16) were introduced into each solution. All test solutions and the control solution (containing no active substance) was left under stirring for 24 h. The number of dead larvae in each solution, counted under a microscope, was used to evaluate the toxicity of the solution. In the case where there was death in the control medium, the data was corrected by Abbott's equation [35]:

Dead larvae [%] = $[(n_{test} - n_{control}) / n_{control})] \times 100$ [35],

where n_{test} and $n_{control}$ are the numbers of dead larvae in the test and control solutions, respectively. The LC_{50} values were determined by linear regression after the logarithmic transformation of the dose-response data [36]. 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) [37].When a significant difference was observed at the level of 5%, the test of Newman-Keuls was used to separate the averages [38].

RESULTS AND DISCUSSION

Phytochemical screening

Investigations on the phytochemical screening of *O. gratissimum* leaves, seeds and stems extracts revealed the presence of polyphenolic compounds (catechic and gallic tannins, flavonoids, anthocyanins, leucoanthocyans) of quinone derivatives, triterpenoids and steroids; mucilages, coumarins, reducing compounds and essential oils. Recently, a phytochemical analysis of the leaf extracts of the plant from India revealed the presence of potentially antimicrobial active agents such as alkaloids, phenolics, glycosides, resins, steroids, and tannins [39]. Ighodaro et al. [40] have previously purified a fraction rich in flavonoids in plants acclimated in Nigeria. Salu et al. [41] show that the phenolic fraction of the plant from Nigeria have an in *vitro* antioxidative actions. This confirms the presence of polyphenolic compounds in the plant. The contents of polyphenolic compounds, quinons, steroids, coumarins and mucilages depend on the part of the plant studied. Triterpenoids and reducing compounds were present in all parts of the plant, but less abundant. Whatever the vegetative stage, a compound found in a part of the plant was also in the

others. The chemical compositions of the different parts of the plant in full and pre-flowering stages seemed qualitatively identical and quantitatively different. These compounds are known to be biologically active and therefore aid the antimicrobial activities of *O. gratissimum*. These secondary metabolites exert antimicrobial activity through different mechanisms. Tannins have been found to form irreversible complexes with proline-rich protein [42] resulting in the inhibition of cell protein synthesis. Parekh and Chanda [43] reported that tannins are known to react with proteins to provide the typical tanning effect which is important for the treatment of inflammed or ulcerated tissues. Herbs that have tannins as their main components are astringent in nature and are used for treating intestinal disorders such as diarrhea and dysentery [44]. The presence of tannins in *O. gratissimum* supports the traditional medicinal use of this plant in the treatment of different ailments.

Another secondary metabolite compound observed in the leaves, seeds and stems extract of *O. gratissimum* was steroidal compounds. Those are important and interesting due to their relationship with various anabolic hormones including sex hormones [45]. Quinlan et al. [46] worked on steroidal extracts from some medicinal plants which exhibited antibacterial activities on some bacterial isolates. Neumann et al. [47] also confirmed the antiviral property of steroids. Flavonoids, another constituent of *O. gratissimum* leaves, seeds and stems extracts exhibited a wide range of biological activities like antimicrobial, anti-inflammatory, anti-angionic, analgesic, anti-allergic, cytostatic and antioxidant properties [48]. The presence on *O. gratissimum* of non-volatile secondary metabolites with potential pharmacological properties had conduced us to their study.

Table 1: Results of phytochemical screening

Chemical groups	Pre-flowering		Full flowering		
	Leaves	Stems	Leaves	Stems	Seeds
Alkaloids	-	-	-	-	-
Gallic tannins	++++	++	++++	++	+++
Catechic tannins	+++	+++	+++	+++	+++
Flavonoids	++	+++	+++	+++	+++
Anthocyanes	+	++	++	++	++
Leucoanthocianes	++	++	++	++	++
Quinonic derivatives	++	+++	++	++	++
Saponins	-	-	-	-	-
Triterpenoids	+	+	+	+	+
Steroids	++	+	+++	+	++
Cyanogenetic glycosides	-	-	-	-	-
Mucilages	+++	++	+++	++	+++
Coumarins	++	++	++	++	++
Reducing sugar	+	+	+	+	+
Athracenic free	-	-	-	-	-
Athracenic o-heterosids	-	-	-	-	-
Athracenic c-heterosids	-	-	-	-	-
Cardenolides	-	-	-	-	-
Essentials oils	+++	++	+++	++	++

-(absent or not reveled) ; + (present) ; ++ (abundant) ; +++ (more abundant)

Table 2 : Yields of ethanol crude extracts

Samples	Pre-flowering		Full flowering		
	Leaves	Stems	Leaves	Stems	Seeds
***Yields (g*/100g)**	4.81±0.33	1.97±0.05	3.44±0.39	2.06±0.12	2.66±0.32

Table 3: Toxicity towards A. salina larvae and index of selectivity

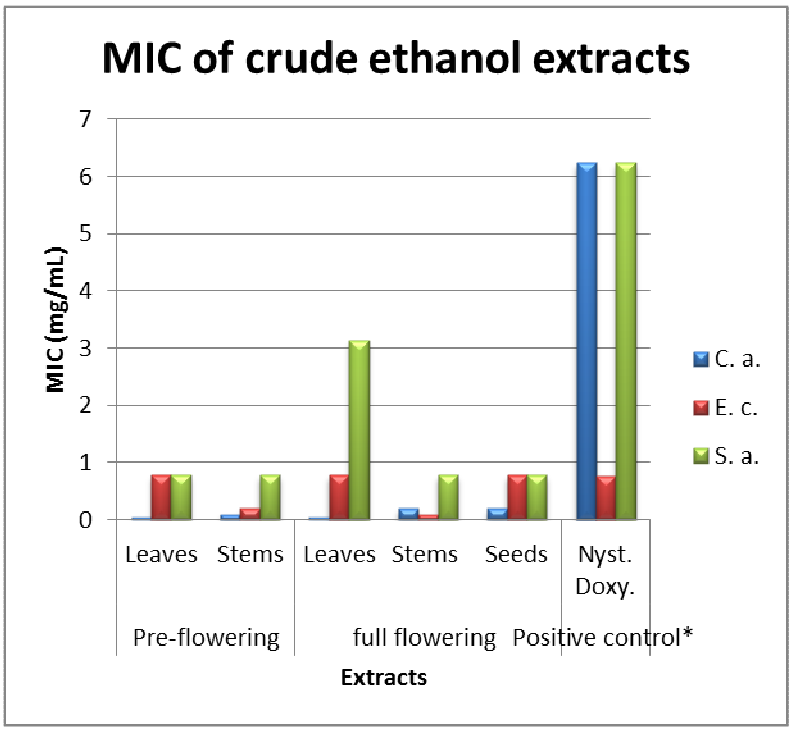
Extract (ethanol)		Pre-flowering		Full flowering		
		Leaves	Stems	Leaves	Stems	Seeds
LC_{50} (µg/ml)		56.1±0.02	102.9±0.02	179.3±0.02	134.2±0.03	284.0±0.02
Selectivity Index[a] **LC_{50}/CMI**	[b]***C. a.***	11.43	10.51	36.53	6.87	14.62
	[c]***E. c.***	0.72	5.28	2.29	13.67	3.65
	[d]***S. a.***	0.72	1.32	0.001	1.72	3.65

[a]Selectivity index = LC_{50}/MIC(C.a. or E.c. or S.a.), [b]C. albicans ATCC, [c]E. coli ATCC, [d]S. aureus ATCC

Table 4: MIC (MB/FC° (mg / mL) of fractions and crude most active ethanol extract of *O. gratissimum*

Germe		Crude extract	Fraction					Positive control (µg/ml)*	Part of plant in full flowering
		Ethanol	Hexane	Chloroforme	Ethyl acetate	Butanol	Water		
[b]C.a.	*MIC*	0.049±0.002	0.077±0.015	0.098±0.012	0.039±0.013	0.225±0.25	0.312±0.013	6.24±0.01	Leaves
	(MFC)	>3.82	>3.80	>3.92	>3.80	>3.87	>3.80	6.24±0.00	
	[e]*R*	>77	>49	>40	>97	>17	>12	1	
[c]E. c.	*MIC*	0.098±0.004	0.156±0.007	0.176±0.027	0.078±0.003	0.878±0,125	2,501±0,109	0.77±0.02	Stems
	(MBC)	>3.92	2.56±0.007	2.512±0.027	1.230±0.003	>3.80	>3.87	0.77±0.01	
	[e]*R*	>40	16.41	14.27	15.77	>4	>0.01	1	
[d]S. a.	*MIC*	0.781±0.031	0.312±0.012	0.412±0.052	0.212±0.012	0.535±0.050	0.625±0.050	6.24±0.01	Stems
	(MBC)	>3.80	>3.80	3.412±0.052	1.230±0.013	>3.82	>3.80	0.77±0.02	
	[e]*R*	>4	>12	8.28	5.80	>7	>6	0.12	

*[b]C. albicans ATCC, [c]E. coli ATCC, [d]S. aureus ATCC; *Nystatin was used for positive control for C. albicans and Doxycycline for positive control for E. coli and S. aureus; [e]R = MFC/MIC or MBC/MIC*



*C. a. = C. albicans ATCC, E. c. = E. coli ATCC, S. a. = S. aureus ATCC;*Nystatin (Nyst.) was used for positive control for C. albicans and Doxycycline (Doxy.) for positive control for E. coli and S. aureus*

Figure 1: Comparison of MIC of ethanol crude extracts of *O. gratissimum* Linn to Nystatin and Doxycycline

Comparison of yields of ethanol crude extracts

The ethanol crude extracts, obtained by percolation procedure of samples of *O. gratissimum*'s leaves, stems and seeds in pre and full flowering gave the highest yields (Table 2). The yields of ethanol crude extracts varied between 1.97% and 4.81%, depending on the vegetative stage and the plant part (table 2). With the same extraction procedure, leave 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)

Identification of the most active and less toxic crude extracts

Ethanol crude extracts were tested for their antimicrobial activities on *C. albicans* ATCC, *E. coli* ATCC and *S. aureus* ATCC (Figure 1). The minimum inhibitory concentrations (MICs) showed that all the crude extracts inhibited the different studied pathogens. For *C. albicans* ATCC, MICs varied between 0.049 and 0.195 mg / mL and the most active extract was from the leaves whatever the vegetative stage. *E. coli* ATCC was sensitive to various extracts with MICs ranging from 0.098 to 0.781 mg / mL. The extract of *O. gratissimum* Linn stems collected in full flowering stage was the most inhibitor of this bacterium. The Benin specie of *O. gratissimum* was

more active on this bacterium than the one of India that showed a MIC value of 2.50 mg/mL [39]. As for *S. aureus* ATCC, the ethanol extracts of leaves and stems in pre-flowering and those of stems and seeds in full flowering showed the same MIC (0.781 ± 0.031 mg / mL). The ethanol extract of the leaves in full flowering gave a lower activity (MIC = 3.125 ± 0.125 mg / mL). These results prove that the vegetative stage and the plant part influence the antimicrobial properties of *O. gratissimum* Linn.

To assess the toxicity towards *A. salina* larvae, LC_{50} values for the various extracts were determined (Table 3). The test used was a preliminary method of estimation of the *in vitro* toxicity of the extracts, 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) [49], A-549 (lung carcinoma), and HT-29 (colon carcinoma) cells [50]. Moreover, Meyer et al. [51] suggested that compounds could be regarded as toxic, if the LC_{50} value is inferior to 30 mg/mL. In our study, the LC_{50} values ranged between 56 and 284 µg/ml. We thus hypothesize that the samples may be considered as not toxic. Also, the selectivity index (SI) of almost all active samples was greater than one except for the leaves extract in full flowering. These extracts (with their SI >1) turn out quite selective on the germs. These results are in perfect agreement with the work of Tiuman *et al*., [52] in which if the SI value obtained is greater than unity, the tested sample is considered to be selective on the parasites and if SI value is less than unity, the test compound is more cytotoxic than antimicrobial.

The minimum bactericidal concentrations (MBCs) were unable to be determined at the concentrations of the work. Different tested crude extracts were thus fongiostatic on *C. albicans* ATCC and bacteriostatic on *E. coli* ATCC and *S. aureus* ATCC. So we decided to continue the fractionation of the actifs crude extracts.

Bio-guided fractionation of the actives crude extracts.

The ethanol crude extract of the leaves and that of the stems of *O. gratissimum* Linn in full flowering, were fractionated and tested for their antimicrobial activities on the studied germs (Table 4). The minimum inhibitory concentrations (MICs) of the fractions: hexane, chloroform, ethyl acetate, n-butanol and aqueous obtainded from the ethanol extract of *O. gratissimum* leaves harvested in full flowering, were evaluated against *C. albicans* ATCC. Those of the same fractions from *O. gratissimum* stems harvested in the same vegetative stage, have been evalueted on *E. coli* ATCC and *S. aureus* ATCC. The ethyl acetate fractions are those that gave the lowest MIC with MIC (0.039 ± 0.013) mg / mL for *C. albicans* ATCC, MIC (0.078 ± 0.003) mg / mL for *E. coli* ATCC and MIC (0.212 ± 0.012) mg / mL for *S. aureus* ATCC (Table 4). These fractions of ethyl acetate were the most active so most concentrated in compounds that inhibit studied pathogens.

Minimum bactericidal concentrations (MBC) of hexane, chloroform, ethyl acetate, butanol and aqueous fractions obtained from ethanol crude extract of leaves and stems of *O. gratissimum* Linn harvested in full flowering, were evaluated on *C. albicans ATCC, E. coli* and *S. aureus* ATCC (Table 4). Contrary to crude extracts which MCB couldn't be determined at the tested concentrations, some fractions gave MCB on *E. coli* and *S. aureus* (Table 4). This proves that the fractionation allowed concentration of bactericidal compounds. To better appreciate the antimicrobial properties of the tested fractions, the ratio MBC/MIC (or MFC/MIC) [33] was calculated for each oil (Table 4), to indicate a bactericidal (MBC/MIC or MFC/MIC < 4) or bacteriostatic (MBC/MIC or MFC/MIC > 4) activity [53]. All the fractions may be considered as fungistatic against *C. albicans* and bacteriostatic against *E. coli*. and against *S. aureus*. According to the results obtained, the properties of *O. gratissimum* need to be further investigated by isolation and identification of pure bioactive compounds by bio-guided fractionation.

CONCLUSION

Our study showed that different samples (leaves, stems and seeds in pre-and full flowering) of *O. gratissimum* contained polyphenolic compounds (gallic and catechic tannins, flavonoids, anthocyanes, the leucoanthocyanes), quinone derivatives, triterpenoids, steroids, mucilage, coumarins, reducing compounds and essential oils. Leaves gave more ethanol extract than seeds and the lower yield was obtained with the stems. Crude extract of leaves and stems in full flowering was the most active respectively against *C. albicans* ATCC, and *E. coli* ATCC and *S. aureus* ATCC with a good selectivity. Ethyl acetate fractions was the most active and needs to be further investigated by isolation and identification of pure bioactive compounds by bio-guided fractionation. This is the first report of interaction between plant parts, vegetative stages, antimicrobial properties and toxicity of the non-volatile fractions of *O. gratissimum* Linn from Benin.

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