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In vitro activities of aqueous and hydro-ethanolic extracts of *Ocimum gratissimum* on *Escherichia coli* ESBL, *Klebsiella pneumoniae* ESBL and methicillin-resistant *Staphylococcus aureus*

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The misuse of antibiotics plays a role in increasing of antimicrobial resistance. The plants, of traditional use, then become an alternative solution to deal with this public health issue. *Ocimum gratissimum* L. is a widely used plant in Togo for several diseases. The objective of this work was to evaluate the antibacterial activity of the hydro-ethanolic and aqueous extracts of the leaves of this plant, by measuring the diameter of inhibition, from the method of the wells on agar medium and the determination of the minimum inhibitory concentration (MIC), and the bactericidal (MBC) concentration. For this purpose, clinical strains of *Escherichia coli*, *Klebsiella pneumoniae* and *Staphylococcus aureus* were used. The inhibition diameters were between 10.00 ± 0.58 and 14.67 ± 0.58 mm, and the MICs between 6.25 and 25.00 mg/ml. The results obtained show that the hydro-ethanolic and aqueous extracts have been active on all the strains tested with a bactericidal activity on the majority. However, the hydro-ethanolic extract was significantly more effective than the aqueous extract (P = 0.049) on all strains. This work has provided us a scientific basis of the use of *O. gratissimum* L. in our environment, particularly for the treatment of bacterial infections.

Key words: *Ocimum gratissimum L*, hydro-ethanolic and aqueous extracts, antibacterial activity, multi-resistant strains, Togo.

INTRODUCTION

In developing countries, infectious diseases are a public health problem because of their frequency and severity. Indeed, they cause more than 17 million deaths a year in the world, more than half of which come from the African continent alone (WHO, 2006). The discovery of antibiotics was a real revolution in the fight against infectious diseases. However, make improper use and abuse of antibiotics have accelerated the emergence of multi-resistant bacteria, which is now a real problem of antibiotics and public health (Salou et al., 2016).

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The use of natural resources in general and medicinal plants in particular becomes one of the most important and interesting avenues for the search for new and more effective antibacterial products (Chebaibi et al., 2011). Medicinal plants are known to contain substances which could be used for treatment purposes or used to produce drugs (Udochukwu et al., 2015). Plants have long been an inspiration for the search for new medicines, contributing to the well-being and health of humans (Amengialue et al., 2013).

Plants have been used by all cultures throughout history and thus, herbal medicine is the oldest form of health care known to mankind. It was an integral part of development of modern civilization (Ojo et al., 2013). Indeed, in developing countries, almost 80% of the rural population use medicinal plants to treat themselves (WHO, 2011). This is due to the efficacy, accessibility, availability, low toxicity and tolerance of plants (Akharaiyi and Boboye, 2010).

Today, the permanent resistance of certain bacteria to classical antibiotics leads to the search for new active ingredients (medicines) based on plants as sources of compounds that can overcome the cases of resistance in order to put under control new infections (Akharaiyi and Boboye, 2010). It is within this framework that the present study aims to evaluate the antibacterial activity of strains of *Escherichia coli*, *Staphylococcus aureus* and *Klebsiella pneumoniae*. These 3 bacterial strains are frequently isolated routinely in our labs and they presented a high level of antibiotics resistance.

Ocimum gratissimum on which has pharmacological properties such as antibiotic properties, antidiarrheal (Kpodekon et al., 2013), antifungal (Soro et al., 2011), antihyperglicemic (diabetes) (Koane et al., 2011), anesthetic, cicatrizing and stimulating is widely consumed in Togo as a food and medicinal plant in the treatment of bacterial infections.

This study was to give a scientific basis for the traditional use of this plant against infections in Togo.

MATERIALS AND METHODS

Collection of plant

The plant material consists of *O. gratissimum* leaves purchased from the Akodessewa market and identified at the Herbarium of the Botanical and Plant Ecology Laboratory of the Faculty of Science at the University of Lomé, Togo.

Collection of micro organism

The bacterial strains were composed of the strains of *S. aureus* ATCC29213 for Gram positive and *E. coli* ATCC25922 bacteria, *K. pneumoniae* ATCC13883 for Gram negative bacteria and multi-resistant strains of *S. aureus* (MRSA), *Escherichia coli* (ESBL) and *K. pneumoniae* (ESBL) isolated from bacterial specimens in patients. The Laboratory of Bacteriology of the National Institute of Hygiene (NIH), in Togo, supplied these strains.

A previously conducted antibiogram showed the profile of each strain used in this study. These organisms were selected because of their frequency in bacterial infections.

Preparation and extraction of the plant

The leaves of *O. gratissimum* were dried at laboratory temperature, protected from bright light and pulverized in the mill. 200 g of plant powder was macerated in 2 L of distilled water and ethanol-water (70:30) for 72 h (Zirihi et al., 2003; Kouadio et al., 2015). The filtration was carried out on Whatman No. 1 paper and the filtrate obtained was evaporated using Rotavapor. The extracts obtained were filtered through a millipore membrane and the filtrate, stored in a refrigerator at 4°C (Zirihi et al., 2003; Kouadio et al., 2015). The extract was seeded on a Petri dish containing nutrient agar and incubated at 37°C for three days with observation every 24 h to verify the sterility test of the extract. The extract was revealed sterile if no colony is visible on the agar box.

Susceptibility testing

The inoculum was prepared from a 24-h young colony, emulsified in 10 ml of physiological water having an optical density adjusted to 0.5 Mac Farland using a densimat.

The antibacterial activity was carried out by the solid-state diffusion method (Dosso and Faye-Kette, 2000; Koné et al., 2004; Kouadio et al., 2015). Petri dishes containing Muller-Hinton agar were inoculated with the inoculum. Then, wells excavated in the agar were filled with 50 µl of the extract solution (100 mg/ml). The whole was incubated at 37°C for 24 h. The inhibition diameter around each well was measured with a caliper; the test was performed in Triplicata. The efficacy of the extracts was evaluated according to the criterion of Ponce et al. (2003).

Determination of MIC and MBC

The determination of MIC and MBC was performed using the liquid-phase macro-dilution method using hemolysis tubes (Konan et al., 2013; Kouadio et al., 2015). From a stock solution of 100 mg/ml extract, a successive dilution of reason 2 (100-1.56 mg/ml) is carried out with Mueller-Hinton broth and a microbial suspension broth at 10^5 Mac Farland. Thus, in experimental haemolysis tubes, 1 ml of each plant extract concentration range was contacted with 1 ml of bacterial inoculum. The growth control tube received 1 mL of sterile Mueller-Hinton broth in addition to the inoculum while the sterility control received only Muller-Hinton Broth. The tubes thus seeded are covered with carded cotton and incubated 24h at 37°C. The next day, the tube corresponding to the lowest concentration of extract for which no turbidity is observed, is taken as the minimum inhibitory concentration (MIC) of the extract on the strain tested. Minimum bactericidal concentration (MBC) is the concentration of a substance which, after 24 h incubation at 37°C, gives 0.01% viable bacteria. His determination began with numeration. This consisted of diluting the starting inoculum from 10^{-1} to 10^{-4} and inoculating these various dilutions with the aid of a 2 µl calibrated loop in 5 cm long strips on a Muller-Hinton agar (MHA) and incubating for 24 h. These Petri dishes were named A. After MIC reading, the contents of the tubes in which there was no visible growth was used to inoculate the Muller-Hinton agar on 5 cm striations. The incubation was carried out for 24 h. This series of Petri dishes is named B. The MBC was determined by comparing the bacterial growth of the A and B boxes. Thus, the lowest concentration of the tube which has less than 0.01% viable bacteria relative to inoculum is the MBC. The MBC/MIC report made it possible to specify the modality of action of a substance (Fauchere and Avril, 2002). If the MBC/MIC

Table 1. Diameters of inhibition zones of *Ocimum gratissimum* leaf extracts and antibiotics on bacteria.

Germ	Codes	Profils	Diameters of inhibition(mm)				p-Value
			HEE*	AE**	DW***	FEP****	
E. coli	ATCC25922	Wild	11.67±0.58	10.33±0.58	6.0±0.0	29.5±0.71	0.02
E. coli	2860/16	ESBL	11.33±0.58	10±0.58	6.0±0.0	14.75±0.35	0.02
K. pneumoniae	ATCC13883	Wild	14.67±0.58	12.33±58	6.0±0.0	29±1.41	0.00
K. pneumoniae	1283/16	ESBL	13.33±0.58	11.33±58	6.0±0.0	14.75±0.35	0.00
S. aureus	ATCC29213	Wild	14.67±0.34	12.33±0.58	6.0±0.0	28±1.41	0.01
S. aureus	0990/16	MRSA	13.67±0.58	11.67±0.58	6.0±0.0	17±0.00	0.01

*Hydro-ethanolic extract, **Aqueous extract, ***Distilled water, ****Cefepim, *Cefoxitin.

Table 2. Antibacterial parameters of extracts of *Ocimum gratissimum* leaves on different strains.

Germ	Codes	Profils	Hydro-Ethanolic extract			Modality
			MIC (mg/ml)	MBC (mg/ml)	MBC/MIC	
E. coli	ATCC25922	Wild	12.50	25.00	2.00	Bactericidal
E. coli	2860/16*	ESBL	25.00	50.00	2.00	Bactericidal
K. pneumoniae	ATCC13883	Wild	6.25	25.00	4.00	Bacteriostatic
K. pneumoniae	1283/16*	ESBL	25.00	50.00	2.00	Bactericidal
S. aureus	ATCC29213	Wild	12.50	25.00	2.00	Bactericidal
S. aureus	0990/16*	MRSA	25.00	25.00	1.00	Bactericidal

*Laboratory identification code.

ratio is less than or equal to 2, the substance is said to be bactericidal. On the other hand, if it is greater than 2, the substance is called bacteriostatic.

The results of microbiological analyses were analyzed using the Excel and Epi info version 6.4 for software to compare the activity of *O. gratissimum* leaf extracts between them on one hand and the activity of extracts on the different strains. The results obtained were expressed as mean ± standard deviation (SD). The level of significance is 5% (p =0.049).

RESULTS

The evaluation of the antibacterial activity of the aqueous-alcoholic and aqueous extracts of *O. gratissimum* at the concentration of 100 mg/ml in agar medium was evaluated by measuring the inhibition diameters.

The hydro-alcoholic and aqueous extracts are effective on all the strains studied. The inhibition diameters are recorded in Table 1.

With regard to the antibacterial parameters, the turbidity induced by the growth of the bacteria decreased inversely with the concentration of extracts in the experimental tubes.

This made it possible to deduce the MICs and to determine the MBCs of the different extracts compared to the germs studied. The results of these different parameters were collected in relation to the different extracts (Table 2).

The data obtained from the study of the various parameters (MIC, MBC) relating to the various extracts made it possible to conclude on the efficacy of the *O. gratissimum* extracts (Table 3).

DISCUSSION

Increasing resistance to antibiotics is the main factor behind the need to identify or develop new antimicrobial agents. Plants have long been used in medicine to treat infections. Thus, plants, in addition to their nutritional potential, would be useful as a drug (Cowan, 1999). In this work, we evaluated the antibacterial activity of the hydro-ethanolic and aqueous extracts of *O. gratissimum* leaves on the *in vitro* growth of wild-type bacteria and those with a multi-resistance phenotype.

Aqueous and hydro-ethanolic extracts were used in relation to the habits of the populations that use this plant, for food needs, but also for health needs. They go through maceration, decoction or infusion. The inhabitants of the Brazilian rainforest use a decoction of *O. gratissimum* roots as a sedative for children (Cristiana

Table 3. Antibacterial parameters of *Ocimum gratissimum* leaves extracts on different strains.

Germ	Codes	Profils	Aqueous extract			Modality
			MIC (mg/ml)	MBC (mg/ml)	MBC/MIC	
E. coli	ATCC25922	Wild	25.00	50	2	Bactericidal
E. coli	2860/16	ESBL	25.00	50	2	Bactericidal
K. pneumoniae	ATCC13883	Wild	25.00	50	2	Bactericidal
K. pneumoniae	1283/16	ESBL	25.00	50	2	Bactericidal
S. aureus	ATCC29213	Wild	25.00	50	2	Bactericidal
S. aureus	0990/16	MRSA	25.00	50	2	Bactericidal

et al., 2006). The cup method used for the determination of the inhibition diameters has the advantage of diffusing the total amount of extract into the agar while one part is retained in the case of blotting discs (Kamanzi, 2002). Inhibition diameters ranged from 11.33 ± 0.58 to 14.67 ± 0.58 mm for hydro ethanolic extracts and between 10.00 ± 0.00 and 12.33 ± 0.58 mm for the aqueous extracts. Mbajiuka et al. (2014) had found diameters of 13 and 15 mm for the hydro-ethanolic extract and for the aqueous extract the diameters were between 7 and 8 mm. Nweze and Eze (2009) reported in their study diameters between 0 and 19 mm. In Nigeria, Salawu et al. (2011) obtained lower diameters ranging from 0 to 6.1 mm for strains of *E. coli* and *S. aureus*. The differences observed in the diameters are due to the difference in the concentrations of the extracts used as is the case in the Salawu et al. (2011) study. Indeed, these authors used a concentration of 50 mg/ml, lower than ours; on the other hand, the differences would come from the method used: the cup method was used in our study while that of the blotting discs was used in the study of Mbajiuka et al. (2014) and Udochukwu et al. (2015) also used the disk method and found inhibition diameters of 7.5 mm for aqueous and hydro-ethanolic extracts of *O. gratissimum* on *E. coli*.

In this study, the strains which were most sensitive to extracts were those of *K. pneumoniae* and *S. aureus*. These results agree with those of Nweze and Eze (2009) who have shown in their study that the hydro-ethanolic extract of *O. gratissimum* had activity on *S. aureus* with an inhibition diameter of 19 mm. According to Nweze and Eze (2009) and Salawu et al. (2011), the extract from *O. gratissimum* showed no activity on *E. coli*. On the other hand, Udochukwu et al. (2015) showed that the hydro-ethanolic extract of the leaves of this plant had an inhibitory activity on *Escherichia coli* and *S. aureus*.

For *K. pneumoniae*, our findings differed from those of Alo et al. (2012), who found that extracts of *O. gratissimum* had no activity. But Alo et al. (2012) have, like us, found an activity of this plant on the *E. coli* strains with inhibition diameters of 20 and 21 mm, respectively for the aqueous extract and for the hydro-ethanolic extract.

The hydro-ethanolic and aqueous extracts of this plant are bactericidal on the majority of strains studied with MICs between 6.25 and 25.00 mg/ml. These results differ from those of Sajini et al. (2016) who found MICs between 0.117 and 7.5 mg/ml. This difference could be due to the solvent used, which in their study was methanol, while in ours we used the aqueous extract and the hydro-ethanolic extract. This study shows that the hydro-ethanolic and aqueous extracts were active on all strains tested and are, respectively bactericidal on 83.33 and 100% of the strains.

The hydro-ethanolic and aqueous extracts of *O. gratissimum* showed activity on the different bacteria studied. However, the hydro-ethanolic extract was significantly more effective than the aqueous extract ($p = 0.049$) on all strains; the same results were found by Nwinyi et al. (2009) and Udochukwu et al. (2015). The active ingredients of the plant are more extractable in ethanol (Kouadio, 2013).

Traditionally, *O. gratissimum* is used in the treatment of several pathologies of bacterial origin such as skin infections, diarrhea and urinary infections (Oussou et al., 2004; Offiah and Chikwendu, 1999; Prabhu et al., 2009). The biological properties of this plant make it possible to justify the antimicrobial activities shown in this study and to relate these activities to the traditional use of *O. gratissimum* in the treatment of pathologies of bacterial origin.

Conclusion

This study demonstrates the antibacterial activity of *O. gratissimum* leaves on *E. coli* strains producing extended spectrum beta-lactamase (ESBL), *K. pneumoniae* producing ESBL and *S. aureus* resistant (MRSA) and also to determine MIC and MBC. This activity is essentially bactericidal in nature. These results justify the traditional use of this plant in the treatment of certain diseases of bacterial origin in Togo and thus give it a scientific basis.

It would therefore be interesting to undertake toxicity studies of this plant and to consider the development of short-term improved traditional medicines.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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