Susceptibility Patterns in *Staphylococcus* and *Klebsiella* Causing Nosocomial Infections upon Treatment with E-Anethole-Rich Essential oil from *Clausena anisate*

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Article

Susceptibility Patterns in *Staphylococcus* and *Klebsiella* Causing Nosocomial Infections upon Treatment with *E*-Anethole-Rich Essential oil from *Clausena anisate*

François Nguimatsia [1], Evariste Josué Momo [2,3], Paul Keilah Lunga [4], Virginia Lum Tamanji [1], Boniface Pone Kamdem [4,*] and Pierre Michel Jazet Dongmo [3]

[1] Laboratory of Pharmacognosy, Faculty of Pharmacy, University of the Mountain, P.O. Box 208 Bangangté, Cameroon; Email: nguimfranc@yahoo.com (F.N.), tamanjivirginia@gmail.com (V.L.T.),
[2] Higher Teacher Training College, Bertoua (HTTC), University of Bertoua, P.O. Box 652, Bertoua, Cameroon; Email: momoevaj@yahoo.fr (E.J.M.),
[3] Laboratory of Biochemistry, Faculty of Science, University of Douala, P.O. Box 24157, Douala, Cameroon; Email : mjazet@yahoo.com (P.M.J.D.),
[4] Antimicrobial and Biocontrol Agents Unit (AmBcAU), Laboratory for Phytobiochemistry and Medicinal Plants Studies, Department of Biochemistry, Faculty of Science, University of Yaoundé I, Yaounde P.O. Box 812, Cameroon; Email: lungapaul@yahoo.ca (PKL); ponekamdemboniface@gmail.com (B.P.K.).
* Correspondence: ponekamdemboniface@gamil.com; Tel.: (+237) 680 98 76 69.

Abstract: High rates of resistance to antibiotics are associated with healthcare-related infections, thus appealing for the urgent need for effective antimicrobials against these maladies. The present study aims to determine the chemical composition of essential oil (EO) from *Clausena anisata* leaves, and evaluate their antibacterial activity against selected nosocomial bacteria. Fresh leaves of *Clausena anisata* were subjected to a steam distillation using a Clevenger apparatus to afford the essential oil. The phytochemical composition of this oil was analyzed using gas chromatography-mass spectrometry (GC-MS). The essential oil was further tested against selected nosocomial bacteria, including *Staphylococcus* and *Klebsiella* species, among others, using a microdilution method. As a result, 0.77% of essential oil was extracted from fresh leaves of *Clausena anisata*. The GC-MS analysis revealed that the as-prepared essential oil contain E-anethole (70.77%), methyl isoeugenol (13.85%), estragole (4.10%), γ-terpinene (3.33%), myrcene (2.82%) and sabinene (0.77%), with E-anethole being the major constituent. Meanwhile, twenty two compounds were identified in the EO of *Clausena anisata* leaves through gas chromatography. Upon antibacterial test against selected nosocomial pathogens, the *E*-anethole-rich essential oil exhibited minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values ranging from 3.91 to 125 µg/mL and 7.81 to 125 µg/mL, respectively, indicative of a bactericidal orientation of the plant's essential oil (MIC/MBC ratio<4). This novel contribution highlights the scientific validation of the use of *Clausena anisata* leaves in the traditional treatment of various infectious diseases. However, toxicity and pharmacokinetic studies, mechanistic basis of the antibacterial action, and *in vivo* antibacterial experiments of the *E*-anethole-rich essential oil of *Clausena anisata* should be investigated to successfully use this plant in the treatment of infectious diseases.

Keywords: nosocomial infections; antibacterial activity; drug resistance; *Clausena anisata*; GC-MS analysis; essential oil

1. Introduction

Infectious diseases are a group of illnesses caused by specific infectious agents also called pathogens or their toxic products through an intermediate host, vector or inanimate environment [1].

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Among these infectious agents are nosocomial pathogens, which are acquired during the process of receiving health care and that were not present during the time of admission [2]. During hospitalization, patients are exposed to pathogens through different sources, including the environment, healthcare staff, and other infected patients [2,3]. Nosocomial infections occur worldwide both in developed and developing countries and are the cause of high mortality and morbidity. These maladies account for 7% in developed and 10% in developing countries [2]. According to WHO estimates, approximately 15% of all hospitalized patients suffer from these infections [4]. These diseases are responsible for over 4-56% of all death causes in neonates, with an incidence rate of 75% in South-East Asia and Sub-Saharan Afric [2]. The incidence of nosocomial infections is high enough in high income countries, specifically between 3.5% and 12%, whereas the same varies between 5.7% and 19.1% in middle and low income countries [2]. The most frequent types of nosocomial infections include central line-associated bloodstream infections, catheter-associated urinary tract infections, surgical site infections and ventilator-associated pneumonia [5,6]. Among these, surgical site infections and ventilator-associated pneumonia are mainly caused by *Staphylococcus aureus* and *Klebsiella pneumonia*, respectively, resulting in prolonged hospitalization and risk of death in admitted patients [2,7]. Transmission of such germs happens in hospitals, nursing homes, and other places with lots of sick people or through touch of a cut during hand shaking by contact with medical devices [2,8]. The high resistance of *Klebsiella* species (Enterobacteriaceae) to carbapenems, and *Staphylococcus* species to methicillin make these pathogens highly virulent, especially when they travel from the gastrointestinal tract (opportunistic pathogen, *Klebsiella*) to other parts of the body [9,10] or when they are acquired through direct contact with open wounds and contaminated hands (*Staphylococcus aureus*; [2]). The Gram-negative bacteria *Klebsiella*, Enterobacter, and *Serratia* are closely related normal intestinal flora that rarely cause disease in normal hosts. *Serratia* species are Gram-negative bacilli of the Enterobacterales order, although they are not a common component of healthy human fecal flora [11]. However, *Serratia* species may harbor multidrug resistance mechanisms that can complicate treatment resolutions [11,12]. Current treatments for nosocomial diseases include carbapenems (meropenem) and beta lactams (tazobactam and avibactam), as well as combination therapies, such as meropenem–vaborbactam, ceftolozane–tazobactam, imipenem–cilastatin/relebactam, meropenem–vaborbactam, ceftazidime–avibactam and aztreonam–avibactam [13,14]. However, the misuse and overuse of these antimicrobials are the main drivers in the development of drug-resistant pathogens [13,15]. Moreover, a number of adverse effects [(carbapenems: injection site reactions, diarrhea, nausea, coagulation abnormalities, nephrotoxicity, or hepatotoxicity, vomiting, and skin rashes [16,17]; beta lactams: neurotoxicity and nephrotoxicity, as well as hematological adverse events and drug-induced liver injury [18]; cephalosporins: cefepime-induced neurotoxicity [19] have been identified in these therapies. Thus, there is an urgent need to search for safe and effective antimicrobials against nosocomial infections caused by bacteria.

Medicinal plants, which have been the most valuable source of molecules with therapeutic potential, are still an important pool for the identification of new and safe drugs, including antimicrobials [20]. It is even expected that plant extracts showing target sites other than those used by antibiotics will be active against drug resistant pathogens [21].

Various parts of *Clausena anisata* have been reported as effective treatments against parasitic infections, especially flatworm infections, such as taeniasis and schistosomiasis, and eye diseases; influenza and other respiratory illnesses [22]; hypertension; heart diseases; and abdominal cramps. Moreover, other reports indicated *Clausena anisata* as an effective plant against constipation and gastroenteritis, liver disease-induced breathlessness, fevers and pyrexia, malaria, boils, arthritis, rheumatism and other inflammatory diseases, toothaches, body pains, headaches, swollen gums, impotence, convulsions, sterility, and several mental disorders [23]. The leaves of *C. anisata* are used to treat hypertension in South Africa, and fresh leaves are burned to repel mosquitoes in the Philippines [24,25]. For generations, different parts of *Clausena* plants, including root, bark, stem and leaves, as well as essential oil, which are rich in alkaloids, flavonoids, monoterpenes and triterpenoids,

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have traditionally been used to treat a number of diseases, while being a source of drug for modern-day microbial diseases [26].

In previous reports, modern pharmacological studies revealed the antimicrobial activity of the essential oil of *C. anisata* on *Salmonella typhi* and *Pseudomonas aeruginosa* [23]. However, the effect of *C. anisata* against nosocomial infections caused by *Staphylococcus* and *Klebsiella* species, has not yet got scientific evidence. Thus, the present study demonstrates the antibacterial activity of *C. anisata* essential oil against selected nosocomial pathogens, including clinical strains of *Staphylococcus*, *Klebsiella*, and *Bacillus* species, and others.

2. Material and methods

2.1. Material

2.1.1. Plant material

Clausena anisata leaves (Figure 1) were harvested at Bafou, Dschang, Menoua division (West Region of Cameroon). The plant was later identified at the National Herbarium of Cameroon (NHC) in Yaounde where a voucher specimen was deposited under number 2711/SRFK.

Figure 1. Photograph of *Clausena anisata* growing on a fence in Bafou, Dschang, West region, Cameroon (picture by V.L.T.).

2.1.2. Bacterial strains

Table 1 summarizes the bacterial strains used and include nosocomial pathogens, such as Bacillus, *Staphylococcus*, *Klebsiella*, and *Serratia* species, and *Pseudomonas aeruginosa*, *Escherichia coli* and *Salmonella typhimurium* (Table 1).

Table 1. Different bacterial strains.

Species	Strains
Bacillus spp.	3C4 UR Mcc
Staphylococcus aureus	00166 6/14
Pseudomonas aeroginosa	2’
Klebsiella pneumoniae	Rea Ro2Mcc
Staphylococcus epidermis	3
Serratia spp.	2C1 UR CB Mcc
Escherichia coli	2.5922
Salmonella typhimurium	O,6,7 HCN

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2.1.3. Material for bacterial cell culture

In this study, Mueller Hinton Agar was used for the development of the bacterial strains whereas the Mueller Hinton Broth was employed for the determination of the Minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC). These media were obtained from LIOFILCHEM® S.A.R.L. Other reagents included McFarland standard 0.5, sterile distilled water, physiological water (normal saline), tween 80, and anhydrous sodium sulfate.

2.2. Methods

2.2.1. Extraction of the essential oil

The essential oil was extracted from the leaves of *Clausena anisata* using a Clevenger-type apparatus. Briefly, the collected plant material was washed then chopped. Next, the plant material was introduced into a round bottom flask by series of 1 kg for 500 ml of water. The mixture was then brought to boil for a period of 4 to 5 hours. During this process, the vapor undergoes a condensation and divides into 2 phases with the superior phase consisting of the essential oil, which is collected. The water contained in essential oil is then dried using anhydrous sodium sulfate. The oil is further weighed, and the yield is calculated and bottled in a tinted glass 60 ml bottle and refrigerated at 4°C. The yield of the essential oil expressed as a percentage was calculated using the following formula:

$$Y = (Me/Mp) \times 100$$

Where:
Y = yield of essential oil in percentage
Me= mass of essential oil in grams
Mp = mass of plant biomass in grams

2.2.2. Phytochemical analysis

The essential oil obtained was analyzed by gas chromatography (GC) and gas chromatography coupled with mass spectrometry (GC/MS).

For gas chromatography, the oil was injected into a long tubular column, the chromatography column. The chemical constituents of the essential oil were eluted (by steam of helium gas) from the column one after the others, according to their retention time (RT) in the column. Thus, an identifying characteristic of the active principle is the retention time.

Briefly, the oil was analyzed on a Varian CP-3380 GC with flame ionization detector fitted with a fused silica capillary column (30 m x 0.25 mm coated with DB5, film thickness 0.25 µm); temperature program 50°-200°C at 5°C/min, injector temperature 200°C, detector temperature 200°C, carrier gas N2 (1 mL/min). The linear retention indices of the components were relatively determined to the retention times of a series of n-alkanes, and the percentage compositions were obtained from electronic integration measurements without taking into account the relative response factors [27,28].

For the gas chromatography/mass spectrometry analysis, the GC is coupled with a mass spectrometry (MS) detector. As a compound exits from the end of the GC column it is fragmented by ionization and the fragments are sorted by mass to form a fragmentation pattern. Like the retention time (RT), the fragmentation pattern for a given compound is unique and therefore is an identifying characteristic of the compound. It is so specific that it is often referred to as the molecular fingerprint [27].

In brief, gas chromatography/mass spectrometry (GC-MS) analyses was performed using a Hewlett-Packard apparatus equipped with an HP1 fused silica column (30 m x 0.25 mm, film thickness 0.25 µm) and interfaced with a quadrupole detector (GC-quadrupole MS system, model 5970). The column temperature was programmed from 70°-200°C at 10°C/min; injector temperature was set at 200°C. Helium was used as the carrier gas at a flow rate of 0.6 mL/min, whereas the mass spectrometer was operated at 70 eV [28].

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After analysis by GC/GC-MS, the identification of different constituents of the oil was carried out by comparison of retention times and mass spectra with known values reported in the literature [28,29]. For each compound identified, the retention index (kovats retention index) was determined according to the following formula:

$$IK = 100 \left(n + \frac{Tr(x) - Tr(C_n)}{Tr(C_{n+1}) - Tr(C_n)} \right)$$

IK = kovats retention index
Tr (Cn) = retention time of alkane at n atoms of carbons
Tr (Cn+1) = retention time of alkane at (n + 1) atoms of carbons
Tr (x) = retention time for compound x

2.2.3. Antibacterial activity

a. Preparation of microbial inocula

To prepare the microbial inocula, single colonies from 24 hours old bacterial culture on MHA were separately isolated using a sterile loop and aseptically introduced into corresponding test tubes containing 10 mL of sterile normal saline (NaCl 0.9%). The turbidity of each test tube was adjusted to 0.5 McFarland standard equivalent to 1.5 x 108 CFU/mL for bacteria. Microbial suspensions were diluted using Mueller Hinton Broth (MHB), to give final test concentrations of 1 x 106 CFU/mL.

b. Determination of minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs)

The various antimicrobial parameters (MIC and MBC) of the samples were evaluated using the broth microdilution method as previously described in protocol M07-A9 of the Clinical and Laboratory Standard Institute [30], with some modifications.

b.1. Determination of minimum inhibitory concentrations

Herein, the tests were performed in duplicate in sterile 96 well microtiter plates. Initially, 196 µL of Mueller Hinton Broth (MHB) were dispensed into the wells of the first line and 100 µL in the remaining wells. Four microliters (4 µL) of each test sample were added into the first wells and serial two-fold dilutions were made up to the eleventh wells. Next, 100 µL of 1 x106 CFU/mL of bacterial suspensions were added to the wells to obtain a final volume of 200 µL. The medium and the corresponding microbial suspensions constituted the negative control while the sterility control contained culture medium only. Ciprofloxacin was used as the positive control. The final concentrations ranged from 1000 to 0.048 µg/mL for the test samples and from 3.906 to 0.0038 µg/mL for ciprofloxacin. Afterward, the plates were covered and incubated at 37oC for 24 h. Following incubation, 20 µL of freshly prepared resazurin (0.15 mg/mL) were added in all wells and the plates were further incubated in the same conditions for 30 minutes. After this incubation period, the lowest concentration, which showed no visible color change from blue to pink implied an absence of microbial growth and was considered as the minimum inhibitory concentration.

b.3. Determination of the minimum bactericidal concentrations

To determine the micro biostatic (bacteriostatic) or microbicidal (bactericidal) nature of the test samples, their MBCs were evaluated through liquid subculture of the preparations withdrawn from the microplates initially used for the determination of MICs. After incubation of the microplates used for determination of MICs, 25 µL aliquots of inhibitory wells were withdrawn and transferred into corresponding wells of sterile microplates, containing 175 µL of sample-free broth per well. Hence, the quantities of samples in the various wells were diluted 8 times to eliminate their inhibitory effect. The microplates were then covered and incubated at 37°C for 24 h. Following this incubation time, the minimum bactericidal concentration of each sample was determined using resazurin (0.15 mg/mL)

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(Sigma Aldrich) as previously described by Clinical Laboratory Standard Institute [30]. From the MBC and MIC values, the ratio MBC/MIC were calculated to depict the antibacterial orientation of the essential oil prepared from *Clausena anisata*.

2.3. Statistical analysis

To determine the MIC and MBC values, concentration-response curves were designed using the Prism software package 5.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com (GraphPad, San Diego, USA) and data were reported as mean and standard deviation (SD) values obtained from a minimum of three determinations. Non-linear best fit was plotted with SD and 95% confidence interval. All data were expressed as mean ± standard deviation.

3. Results and discussion

3.1. Results

3.1.1. Extraction of the essential oil

The physical characteristics, which were observed on the essential oil are embodied in Table 2. The color of the obtained essential oil was yellowish brown, oily with an aniseed aroma. From 4600 g of fresh leaves of *Clausena anisata*, 23.46 g of essential oil was obtained to afford a yield of 0.51%.

Table 2. Physical characteristics of the essential oil of *C. anisata*.

Physical properties	**Fresh leaves**
Color	Yellowish brown
Consistence	Oily
Odor	Aniseed aroma

3.1.2. Chemical composition

Upon analysis of the essential oil by using gas chromatography, a chromatogram that was generated shows compounds' peaks at different retention times. The values obtained for retention times were used to generate Kovats indices through calculations using the formula highlighted in material and methods' section.

The Kovats index obtained for each peak facilitated the identification of the chemical compounds of the essential oil from *C. anisata* fresh leaves.

Upon GC-MS analysis of the essential oil of *C. anisata* leaves was found to containing E-anethole (70.77%), methyl isoeugenol (13.85%), estragole (4.10%), γ-terpinene (3.33%), myrcene (2.82%) and sabinene (0.77%) (Table 3).

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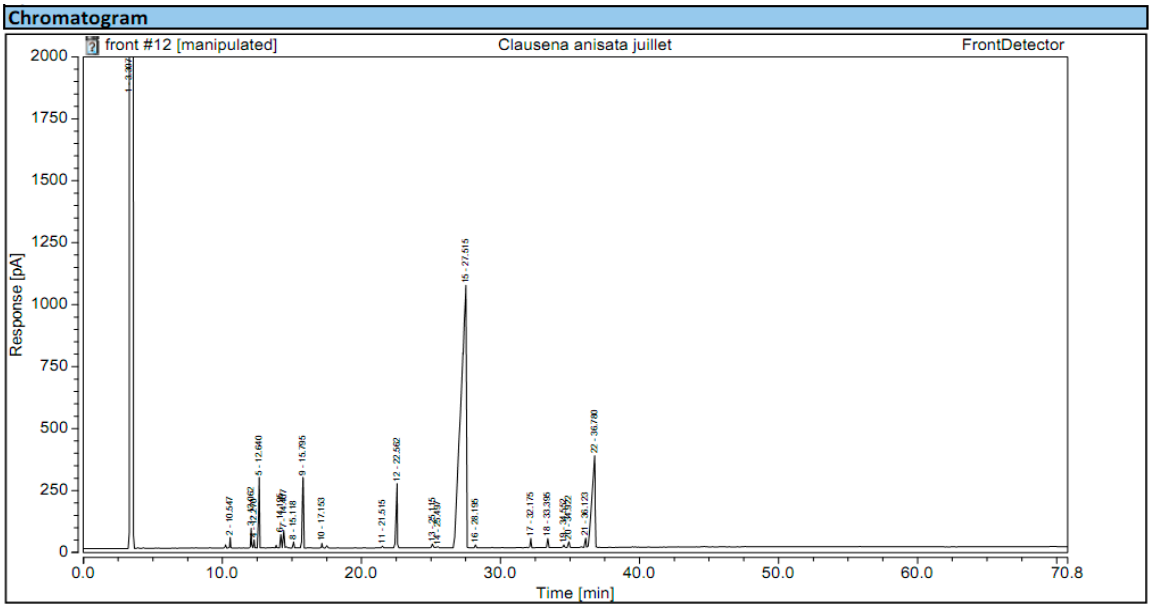

Figure 2. Chromatogram of the essential oil of *Clausena anisata.*

Table 3. Chemical composition of the essential oil of *Clausena anisata.*

Serial N°	RT	IK	Identified compound	Percentage (%)
1	10.547	933	α –Pinene	0.26
2	12.062	971	Sabinene	0 .77
3	12.270	976	β –Pinene	0.26
4	12.640	986	Myrcene	2.82
5	14.195	1021	p-Cymene	0.52
6	14.407	1025	Limonene	0.77
7	15.118	1041	E-β –Ocimene	0.26
8	15.795	1055	γ –Terpinene	3.33
9	17.153	1084	Terpinolene	0.25
10	21.515	1171	α –Terpineol	Nd
11	22.562	1191	Estragole	4.10
12	25.115	1242	para anisaldehyde	0.25
13	25.497	1250	*Z*-Anethole	0.25
14	27.515	1291	*E*- Anethole	70 .77
15	28.195	1305	Cinamyl alcool	Nd
16	32.175	1387	Cinamylacetate	0.52
17	33.395	1413	Methyleugenol	0.52
18	34.552	1438	β –Caryophyllene	Nd
19	34.922	1447	Isoeugenol	0.25
20	36.123	1473	γ –Gurjunene	0.50
21	36.780	1487	Methyl iso eugenol	13.85

IK: Index of Kovats; **Nd:** Not determined; **RT:** Retention time.

3.1.3. Antibacterial activity

The evaluation of the antibacterial activity of the essential oil from *C. anisata* fresh leaves on eight (08) bacterial strains that are incriminated in nosocomial infections led to the determination of the minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) as indicated in Table 4. The incubation of the bacterial strains with the essential oil afforded MIC and MBC values ranging from 3.91 to 125 µg/mL and from 7.81 to 125 µg/mL, respectively. Except for *Pseudomona aeruginosa* and *Salmonella typhimurium*, the essential oil from *C. anisata* leaves was found

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to be bactericidal for all the nosocomial pathogens tested as the calculated MBC/MIC ratios were found to be less than 4 (Table 4). It is well known that natural products (plant extracts and compounds, essential oils, etc.) reveal a bactericidal orientation when the MBC/MIC ratio is less than 4 [31–34]. Consequently, the essential oil from *C. anisata* leaves revealed bactericidal orientation against *Klebsiella* and *Staphylococcus* species, the pathogens that are responsible for most of the nosocomial diseases. Against the *Bacillus* species, the essential oil revealed a common value for MIC and MBC (31.25 µg/mL). Moreover, *Salmonella typhimurium* and *Escherichia coli* were inhibited with a common MIC value of 31.25 µg/mL, whereas the MBC values obtained against these pathogens were respectively 125 and 62.5 µg/mL.

Table 4. Antibacterial activity of the essential oil from *C. anisata* leaves.

Bacterial strains	MIC (µg/mL)	MBC (µg/mL)	MIC/MBC
Bacillus spp.	31.25	31.25	1
Staphylococcus aureus	15.63	15.63	1
Klebsiella pneumoniae	3.91	15.63	4
Pseudomonas aeroginosa	62.5	62.5	1
Staphylococcus epidermidis	3.91	7.81	2
Serratia spp.	3.91	/	/
Escherichia coli	31.25	62.5	2
Salmonella typhimurium	31.25	125	4

MIC: Minimum inhibitory concentration; MBC: Minimum bactericidal concentration; MHB: Mueller-Hinton Broth.

3.2. Discussion

The present study aims to evaluate the antibacterial activity of the essential oil from fresh leaves of *C anisata* against selected nosocomial pathogens. The essential oil was obtained by using a Clevenger apparatus as yellowish-brown oil, with a smell similar to that of the aniseed [35]. Almost similar physical characteristics were obtained by Yaouba et al. [36], while working on the antifungal activity of the essential oil from *C. anisata* leaves. The yield of extraction of the essential oil was 0.77%, which value was found to be superior to that (0.55%) obtained by Okokon in 2012 [37] while working on the essential oil of *C. anisata* leaves collected from Nigeria [37], thus highlighting potential differences in the chemical composition of essential oil from *C. anisata* leaves, which are collected in various areas. Upon analysis of the essential oil from *C. anisata* leaves through GC/MS technique, its chemical constituents included E-anethole (70.77%), methyl isoeugenol (13.85%), estragole (4.10%), γ-terpinene (3.33%), myrcene (2.82%), sabinene (0.77%). This chemical composition of the oil is quite different from that [trans-anethole (69.3 %), methyl isoeugenol (13.2 %), ɣ- Terpinene (4.4 %), myrcene (3.8%), estragole (2.3 %), β- phellandrene (1.2%), β-Caryophyllene (0.8%), germacrene-D (0.7%) and methyl eugenol (0.5%)] obtained by Njonkep in 2014 [38], on *C. anisata* leaves collected in the same area (Bafou, Dschang, West Region of Cameroon). These results suggest the impact of seasons and climatic conditions on the chemical composition of essential oil extracted from plants [38]. The effect of time of plant collection and extraction, as well as extraction conditions on the yield of extraction is noteworthy. In fact, the chemical composition of the essential oil from *C. anisata* leaves differ according to the place of plant harvest [Mount Bamboutos (major constituent: 93.1% of myrtenyl acetate; absence of estragole) [39], Ngaoundere (*C. anisata* leaf essential oil deprived of estragole) [36]. Thus, it can be speculated that the chemical compounds of any plant's essential oil vary greatly depending on the geographical region, the age of the plant, local climatic, seasonal and experimental conditions [40]. Genetic differences are also responsible for the changes of chemical compounds thereby altering the studied biological activities [41]. These differences in the chemical composition of essential oil might significantly impact the antimicrobial efficacy. In this study, the incubation of the bacterial strains with *C. anisata* leaf essential oil afforded MIC and MBC values ranging from 3.91 to 125 µg/mL and from 7.81 to 125 µg/mL, respectively. These results suggested a

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bactericidal orientation of the essential oil as the MIC/MBC ratio was found to be less than 4. A similar trend was reported by several authors [42–44] after determination of MIC and MBC correlations following incubation of selected bacteria with extracts and/or essential oil from plants. Other authors reached similar conclusions while working on the influence of natural and synthetic compounds vis-à-vis kinetic of bacterial mortality as a function of time (time kill kinetics) [44–48]. The observed antibacterial activity might be attributed to the presence of a number of monoterpenes (E-anethole, methyl isoeugenol, myrcene sabinene, and γ-terpinene) in the plant, which was mainly dominated by E-anethole (70.77%). In fact, these compounds are well known for their antibacterial activity, although their mechanism of action is poorly understood. Indeed, in a study by Senatore et al. [49], an trans anethole-rich oil from another plant species i.e. *Foeniculum vulgare* leaves exhibited antibacterial activity against a series of bacterial strains, including *Bacillus subtilis*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus faecalis*, *Escherichia coli*, and *Klebsiella pneumoniae*, etc., attesting the potential of anethole to significantly inhibit the growth of several bacteria. Other studies [50–52] have also revealed the antibacterial activity of trans-anethole against numerous bacterial strains. Previous studies have also demonstrated the inhibitory effect of *E*-anethole, against some fungal strains, such as *Saccharomyces cerevisiae* [50]; however, the antibacterial activity of E-anethole has not yet been reported. Nevertheless, it is speculated that the antibacterial mechanisms of action of these lipophilic compounds involve bacterial membrane disruption [53–56]. The antibacterial activity of monoterpene-rich essential oil has been attributed to inhibition and eradication of biofilm formation [51,57], increase of membrane permeability that allows higher amounts of test samples to enter pathogenic cells and consequentially destroy them [58]. In addition, Li et al. [59] demonstrated the antibacterial activity (MIC values: micromolar range) of a number of monoterpenes isolated from *Illicium simonsii* stems and leaves. Li's group attributed the observed antibacterial activity of *Illicium simonsii* monoterpenes to the disruption of the bacterial membrane permeability as revealed by 4′,6-diamidino-2-phenylindole (DAPI) and propidium iodide (PI) assays [59]. A number of previous reports [60–62] have also pointed out efflux pump as one of the mechanisms by which monoterpene-rich essential oils exert antibacterial activity.

To our knowledge, this is the first report on the inhibitory potential of an E-anethole-rich essential oil from *Clausena anisata* leaves against *Staphylococcus* and *Klebsiella* causing nosocomial infections. The present study validates the traditional use of *Clausena anisata* leaves in the treatment of various infectious diseases.

4. Conclusions

Nosocomial pathogens are responsible for a number of diseases, including catheter-associated urinary tract infections, central line-associated bloodstream infections, skin and soft tissue infections, ventilator-associated and hospital acquired pneumonia, and surgical site infections, with bacteria causing about 90% of these healthcare-associated infections [63]. The treatment of these infections has been hampered by the emergence of drug resistance, thus there is an urgent need to search for effective treatments against nosocomial pathogens. In this study, the antibacterial activity of an E-anethole-rich essential oil from *Clausena anisata* leaves was evaluated against *Staphylococcus* and *Klebsiella* causing healthcare-associated infections. E-anethole-rich essential oil from *Clausena anisata* leaves, which was obtained by steam distillation using a Clevenger apparatus was further analyzed by GC-MS. As a result, the essential oil from *Clausena anisata* leaves (yield of extraction: 0.77%) was found to be majorly dominated by the bicyclic monoterpenoid E-anethole. The as-prepared essential oil inhibited the growth of *Staphylococcus* and *Klebsiella* species with MIC and MBC values ranging from 3.91 to 125 µg/mL and from 7.81 to 125 µg/mL, respectively, suggesting a bactericidal orientation of this essential oil. This novel contribution highlights the scientific validation of the use of *Clausena anisata* leaves in the traditional treatment of numerous infectious diseases. However, toxicity and pharmacokinetic studies, mechanistic basis of the antibacterial action, and *in vivo* antibacterial experiments of the essential oil of *Clausena anisata* are warranted.

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