



Antibacterial effect of ethanolic extract of *Ocimum gratissimum* against *Chromobacterium violaceum*

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Abstract

The use of synthetic antibiotics for managing bacterial infections are increasingly challenged by high costs, limited accessibility, adverse side effects, and the alarming rise in antimicrobial resistance. This resistance has increased the interest of medical researchers in herbal remedies that can serve as alternatives to conventional antibiotics. This study investigated the antibacterial potentials of *Ocimum gratissimum* leaf extract against *Chromobacterium violaceum*. The organism was identified using biochemical and molecular techniques. The susceptibility of *C. violaceum* to conventional antibiotics was done using disc diffusion method. The leaves of *Ocimum gratissimum* were collected, identified and ground. Extraction was done using Soxhlet method with ethanol as the extraction solvent. Phytochemical composition of the extract was analyzed using spectrophotometric method. The agar well diffusion method was used to evaluate the in-vitro antibacterial effect of the extract against *C. violaceum*. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined by broth dilution method and subculturing respectively. The susceptibility of *C. violaceum* to conventional antibiotics showed that Ciprofloxacin gave the highest zone of inhibition (29.83 ± 0.29). Phytochemical analysis revealed that the extract has appreciable levels of alkaloids, flavonoids, phenols, tannins, saponins, phytates and terpenoids. The extract demonstrated concentration-dependent antibacterial activity against *C. violaceum*. At 1000 mg/ml, the extract produced an inhibition zone of 20.67 ± 0.58 mm. The MIC was 62.5 mg/ml while the MBC was 1000 mg/ml. The findings demonstrated that the leaf extract of *O. gratissimum* possessed promising antibacterial activity against *C. violaceum* and may serve as alternative antibacterial agent against *C. violaceum*.

Keywords: *Ocimum gratissimum*, *Chromobacterium violaceum*, extract, phytochemical, antibacterial, Minimum inhibitory concentration, minimum bactericidal concentration

Introduction

Coupled with the scourge of infectious diseases is the recent emergence of drug-resistant microorganisms that have reduced the effectiveness of antimicrobial agents (Montefiore *et al.*, 1989). Among these microorganisms is *Chromobacterium violaceum*, a Gram-negative bacterium commonly found in soil and aquatic environments, which has been implicated in opportunistic and sometimes fatal infections in humans (Alisjahbana *et al.*, 2021). It is a facultative anaerobic free-living bacterium capable of surviving in diverse environmental conditions (Kumar, 2012; Alisjahbana *et al.*, 2021; Lima-Bittencourt *et al.*, 2007^[9]). It is a bacterium found in the environment as a saprophyte (Duran and Menck 2001)^[14]. Reports have shown that *Chromobacterium violaceum* exhibits resistance to several commonly used antibiotics, particularly penicillins and some cephalosporins, highlighting the need to investigate alternative antimicrobial agents, including compounds derived from natural sources (Alisjahbana *et al.*, 2021; Kothari *et al.*, 2017^[22]). The increasing prevalence of antimicrobial resistant strain has further emphasized the importance of exploring plant-based antimicrobials. Seeking remedies for human ailments from the environment has formed the basis for therapeutics (Potier *et al.*, 1990). Synthetic antibiotics, though effective, are often associated with high costs. Consequently, there is a growing interest in identifying natural, cost-effective and eco-friendly alternatives for controlling pathogenic bacteria. Plant

extracts, being biodegradable, affordable and locally available, present a viable option for managing pathogens. According to Bringmann and Pokorny (1995)^[11], African plants constitute a rich untapped pool of natural products. Medicinal plants are of great importance to the health of individuals and communities. Since edible plant products are potential antimicrobial agents, they may be largely preferred because of their widespread availability and effectiveness as bioactive metabolites. The use of these plant materials as spices, condiments and for medicinal purposes dates to the history of mankind (Garland, 1972; Ogunyemi, 1979)^[15, 27]. Plant-derived compounds have attracted attention because of their structural diversity and ability to act through mechanisms that differ from conventional antibiotics (Álvarez-Martínez *et al.*, 2021^[4]). Investigating the phytochemical properties and antibacterial potential of locally available medicinal plants against pathogens is therefore relevant and crucial for public health improvement. Phytochemicals are naturally occurring bioactive compounds in plants that play a significant role in defense mechanisms against pathogens. These compounds have been shown to exert antimicrobial effects through various mechanisms, including inhibition of the activity of enzymes and toxins, damage of the bacterial membrane, suppression of virulence factors, formation of biofilm, inhibition of protein synthesis, and quorum quenching (Ajayi *et al.*, 2016). Bioactive compounds with antibacterial effects need to be explored from local natural

resources. Being new, such compounds may not have the problem of microbial resistance and with some structural modification their activity could be diversified.

A widely studied plant in this regard is *Ocimum gratissimum* (scent leaf). *Ocimum gratissimum* is widely available medicinal plants in Nigeria, traditionally used for the treatment of various ailments, including infections (Sofowora, 2008) [29]. This plant is abundant in tropical regions and is extensively used for its medicinal, nutritional and ethnopharmacological properties. The plant is found throughout the tropics and subtropics, and its greatest variability occurs in tropical Africa and India (Aruna and Sivaranakrishna, 1990) [5]. *Ocimum gratissimum*, a member of the Lamiaceae family, is a medicinal plant rich in phytochemicals. The supposed phytochemical properties of *Ocimum gratissimum* make it promising prospect for the development of plant-based antimicrobial agent (Ugueri *et al.*, 2015) [35].

Therefore, this study is aimed at investigating the antibacterial effect of ethanolic extract of *Ocimum gratissimum* against *Chromobacterium violaceum*. The findings are expected to contribute to the development of alternative antibacterial agent and promote the use of indigenous medicinal plants in combating infections.

Materials and methods

Sample collection

The leaf, *Ocimum gratissimum*, was purchased from Eke Awka market Awka, Anambra State, South-East, Nigeria. The leaf was identified at the Department of Botany, Nnamdi Azikiwe University Awka and given identification number: NAUH/35A.

Preparation of *Ocimum gratissimum* extract.

The leaves of the plants were first separated from the stalk, rinsed with water to remove dirt, air dried at room temperature and ground to powder using an electric blender. Ethanolic extraction was performed by Soxhlet extraction method. Fifty grams of the ground dried leaves were weighed into a thimble. The thimble was placed inside the Soxhlet apparatus. A dried tarred solvent flask was placed in position beneath and 100ml of ethanol added and then connected to the condenser and heated to condensation. The extraction time was a minimum of four hours and was done in four successions. On completion, the extract was placed in an evaporating dish and dried in the water bath at 50°C to concentrate it. The concentrated extract was then scooped into a small sterile container. The container was covered, labeled accordingly and stored in the refrigerator at 4°C and used for further tests. (Kavishankar, *et al.*, 2011).

Phytochemical screening

The phytochemicals in the leaves of *Ocimum gratissimum* were quantified using different methods. Determination of alkaloid was done using the method of Harborne (1998) [17]. The total phenol and flavonoid content were determined using a slightly modified colorimetric method described by Barros *et al.*, (2007). The tannin content was determined using the AOAC method (1995). The phytate content was determined using the method of Young and Greaves (1999) [33]. Saponin content was determined using the method of Hiai *et al.*, (1976) while terpenoid content was determined using method described by Indumathi *et al.*, (2014).

Preparation and standardization of inoculum

Pure culture of *Chromobacterium violaceum* was obtained from Microbiology laboratory, Nnamdi Azikiwe university, Awka, Anambra state, Nigeria. The isolate was sub-cultured on nutrient agar to confirm purity and for identification. A standardized bacterial inoculum was prepared in accordance with the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI M02). A fresh, pure culture of the study isolate was used for inoculum preparation. Three morphologically similar, well-isolated colonies were aseptically picked from the culture plate using a sterile loop and suspended in sterile physiological saline (0.85% NaCl). The suspension was mixed thoroughly to obtain a homogeneous bacterial suspension. The turbidity of the suspension was adjusted to correspond to a 0.5 McFarland turbidity standard spectrophotometrically. The resulting suspension represented bacterial density of 1.0×10^8 CFU/ml. The standardized suspension was used for inoculation of the test medium and subsequent antibacterial susceptibility testing (Cheesbrough, 2010; Clinical and Laboratory Standards Institute (CLSI), 2024).

Antibiotic susceptibility test

Antibiotic susceptibility test against non-Enterobacteriaceae with the protocol defined by Kirby-Bauer was adopted. The bacterial strain was streaked evenly on Mueller-Hinton agar (MHA) plate and after 5 mins, the antibiotic disks were aseptically placed gently on the inoculated agar surface. Agar plates were then incubated at 37 °C for 24 hrs and thereafter, the diameter of the inhibition zone was measured in millimetres with a transparent ruler and reported using zones of inhibition because CLSI M100 does not provide organism-specific disk-diffusion breakpoints for *C. violaceum* (Bauer *et al.*, 1966; CLSI, 2024) [3].

Determination of *in vitro* antibacterial activity of the extract.

Microbial culture of *Chromobacterium violaceum* was used for determination of antimicrobial activity of the extracts. Ten grams of the crude extract was measured into a sterile test tube and 10 ml of 20% dimethyl sulfur oxide (DMSO) was added to dissolve it. This gave a 1000 mg/ml concentration of the extract and this was diluted in two-folds to obtain four different dilutions of the extract: 500 mg/ml, 250 mg/ml, 125 mg/ml and 62.5 mg/ml in addition to the 1000 mg/ml concentration. Mueller Hinton agar was prepared and poured into a Petri dish and kept on the bench at 25°C to solidify. The agar medium was streaked with a loop of twenty-four [24] hour culture of the standardized inoculum of the test organism. Using a sterile cork borer of diameter 6 mm, 4 wells were dug into the agar medium. After 15 mins of organism inoculation, each well was filled with 100 µl of the different concentrations of the crude extract using DMSO as the negative control and standard antibiotics (ciprofloxacin) as positive control. The plates were left for 1 hr at 25°C for diffusion to take place and later incubated for 24 hrs at 37°C. The experiment was performed in triplicates and the inhibition zone diameter recorded as the average of the three replicates (Baron *et al.*, 1994; CLSI, 2024) [3].

Minimal Inhibitory Concentration (MIC)

The Minimum Inhibitory Concentration (MIC) of different extracts was determined by the tube dilution method

according to Hussain *et al.*, (2010) and Baron *et al.*, (1994). In this method, the MIC was determined by carrying out a two-fold serial dilution of the 1000mg/ml stock solution of the plant extracts in nutrient broth to obtain, 500 mg/ml, 250 mg/ml, 125 mg/ml and 62.5 mg/ml. Each dilution in a test tube was inoculated with 0.02 ml of the 24 hrs broth culture of test isolates diluted to 0.5 McFarland standards. Additional test tubes that served as positive control and negative control were set up alongside the tests. All the tubes were incubated at 37°C for 24 hrs. After incubation, the lowest concentration of the extract that inhibited growth of the organism, as detected by lack of visual turbidity (matching the negative growth control) was designated the minimum inhibitory concentration (MIC).

Minimum Bactericidal Concentration (MBC)

This was determined by transferring 0.1ml of the culture broth from each of the MIC test tube that showed no growth by visual inspection and cultured on sterile surface of a solidified MHA plates. These were incubated at 37°C for 24

hrs. The MBC was taken as the lowest concentration of antimicrobial agent that allowed less than 0.1% of the original inoculum to survive or prevented bacterial growth after incubation (Baron *et al.*, 1994; Subhashini and Kantha, 2010^[19]).

Data analysis

Data obtained was statistically analyzed using Statistical Package for Social Sciences (SPSS) version 21 and expressed as mean $\pm$ standard deviation (SD) of triplicate values.

Results

The test result on the study isolate based on its Gram reaction, characteristic violet pigmentation and biochemical profile, which showed positive reactions for oxidase, catalase, citrate utilization, nitrate reduction, and motility and negative reaction for indole, urease, methyl red and Voges–Proskauer tests is presented in Table 1.

Table 1: Biochemical characteristics of *Chromobacterium violaceum*

Test	Observation	Result
Gram stain	Gram-negative bacilli	Positive
Catalase test	Bubble production	Positive
Oxidase test	Purple colour within 30 secs	Positive
Motility	Motile	Positive
Citrate	Blue colour with growth	Positive
Indole test	No red ring after Kovac's reagent	Negative
Methyl Red (MR)	No red colour	Negative
Voges–Proskauer (VP)	No pink/red colour	Negative
Urease test	No pink colour	Negative
Nitrate reduction	Nitrate reduced to nitrite	Positive
Violacein pigment	Purple colonies	Positive
Glucose fermentation	Acid produced	Positive
Sucrose fermentation	No acid production	Negative
Lactose fermentation	No acid production	Negative
Mannitol fermentation	No acid production	Negative
Fructose	Acid produced	Negative
Hydrogen sulfide (H ₂ S) production	No black precipitate	Negative
Gas production	No gas bubbles	Negative

The sensitivity pattern of *C. violaceum* to different commercial antibiotics is shown in Table 2. Ciprofloxacin gave the highest zone of inhibition (29.83 ± 0.29) while Clarithromycin, Erythromycin and Clindamycin showed no zone of inhibition (0.00 ± 0.00).

Table 2: Susceptibility/resistance pattern of *C. violaceum* to different commercial antibiotics.

Antibiotic	Disk potency (μ g)	Inhibition zone diameter $\pm$ SD (mm)
Nitrofuratoin	100	11.17 ± 1.04
Gentamicin	10	20.33 ± 0.58
Ciprofloxacin	10	29.83 ± 0.29
Chloramphenicol	10	16.50 ± 0.50
Ofloxacin	10	18.00 ± 0.00
Clarithromycin	10	0.00 ± 0.00
Pefloxacin	10	23.50 ± 0.50
Ceftriaxone	30	25.00 ± 0.00
Amoxycillin	30	10.83 ± 0.29
Streptomycin	30	24.33 ± 0.29
Erythromycin	10	0.00 ± 0.00
Ampicillin	30	2.43 ± 0.51
Cloxacillin	10	0.60 ± 0.53
Levofloxacin	30	27.67 ± 0.58
Cephalexin	30	0.17 ± 0.29
Clindamycin	10	0.00 ± 0.00

The leaf extract of *O. gratissimum* as shown in Table 3 was observed to have a wide range of bioactive compounds. The extract has high quantity of phytochemical values in total phenols, flavonoids and terpenoids.

Table 3: Phytochemical Constituents (%) of the ethanolic extract of *O. gratissimum*.

Phytochemicals (%)	<i>O. gratissimum</i>
Tannins	0.18±0.01
Total phenols	0.58±0.01
Flavonoids	0.09±0.01
Alkaloids	6.00±0.02
Phytates	0.78±0.05
Terpenoids	2.50±0.00
Saponins	1.10±0.00

The inhibition zone diameter of *Ocimum gratissimum* against *Chromobacterium violaceum* is shown in Table 4. The extracts exhibited antibacterial activity against *C. violaceum*, with different concentrations showing different inhibition zone diameters.

Table 4: Inhibition zone diameter (mm) of the ethanolic extract of *O. gratissimum* against *Chromobacteria violaceum*

Extract concentration	Inhibition zone diameter± SD (mm)
1000 mg/ml	20.67± 0.58
500 mg/ml	18.50± 0.50
250 mg/ml	16.50± 0.50
125 mg/ml	13.33± 0.58
62.5 mg/ml	12.50± 0.50
Ciprofloxacin (10 µg) (Positive control)	23.67± 0.58
10% DMSO (Negative control)	0.00 ± 0.00

Key: DMSO - Dimethylsulphoxide

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the extract is presented in Table 5. The MIC of the extract is 62.5mg/ml while The MBC is 1000 mg/ml.

Table 5: Minimum Inhibitory Concentration and Minimum Bactericidal Concentration of the extract against *Chromobacteria violaceum*

Extract MIC (mg/ml) MBC (mg/ml)
<i>O. gratissimum</i> (ethanolic) 62.5 1000

Key: MIC -Minimum Inhibitory Concentration
MBC -Minimum Bactericidal Concentration

Discussion

The positive oxidase and catalase reactions with positive motility and citrate utilization reactions observed in the present study provide support for the identification of the isolate. The negative indole, urease, methyl red and Voges–Proskauer reactions further contributed to its differentiation from other Gram-negative organisms. Purple colonies associated with violacein production were also observed. The production of violet colonies is particularly important because violacein is a characteristic pigment associated with *C. violaceum*. The presence of this pigment provided an initial phenotypic indication. The biochemical profile obtained in this study (Table 1) is consistent with the characteristic laboratory profile of *C. violaceum* that was subsequently strengthened by molecular characterization. The test organism was identified molecularly as

Chromobacterium violaceum strain 726249W 16S ribosomal RNA gene, partial sequence with Sequence ID: MG938493.1. In the present study, phenotypic identification was complemented by molecular identification, thereby increasing confidence in the identification of the test organism.

The mixed susceptibility pattern demonstrated that *Chromobacteria violaceum* was not uniformly susceptible to the antibiotics tested (Table 2). The sensitivity of the isolate to ciprofloxacin is consistent with the strong activity of ciprofloxacin observed in the subsequent plant-extract experiments, where ciprofloxacin served as the positive control. The observed resistance pattern reinforces the importance of antimicrobial susceptibility surveillance. Antimicrobial resistance has become an important global health challenge, creating a need for new antimicrobial compounds and alternative sources of antibacterial agents hence, the need for this study.

The phytochemical composition of *O. gratissimum* as seen in Table 3 is consistent with recent reviews showing that the plant contains numerous phenolic compounds, flavonoids and terpenoid and other biologically active compounds (Ugbogu *et al.*, 2021; Ashokkumar *et al.*, 2021). The high total phenol, flavonoid and terpenoid contents of the extract is significant because these groups have been widely associated with antibacterial activity. Górniak *et al.* (2019)^[16] reviewed the antimicrobial actions of plant flavonoids and identified several mechanisms through which flavonoids may interfere with bacterial cells. Plant phenolics and terpenes are among the phytochemical groups most frequently associated with bactericidal effects, with membrane disruption being one of the commonly proposed mechanisms as described by Álvarez-Martínez *et al.*, (2021). The presence of these phytochemicals provides a reasonable chemical basis for the antibacterial activities observed in the experiment.

Ethanolic extract of *O. gratissimum* against *C. violaceum* demonstrated antibacterial activity at several concentrations (Table 4). Studies have shown that *O. gratissimum* exhibits broad-spectrum antibacterial activity against several pathogenic bacteria. Aliyu *et al.* (2024)^[3] reported that both ethanolic and methanolic extracts of the plant demonstrated significant inhibitory effects against members of the Enterobacteriaceae family. Ugbogu *et al.* (2021) and Ashokkumar *et al.* (2021)^[6] also reported that *O. gratissimum* has antimicrobial properties. The activity of the extract may be associated with its relatively high total phenolic, flavonoid and terpenoid contents. The positive control, ciprofloxacin, produced an inhibition zone of 23.67 ± 0.58 mm, while 10% DMSO produced no inhibition. The absence of inhibition by DMSO indicates that the observed antibacterial activity was attributable to the plant extracts rather than the solvent. The result also demonstrated a concentration-related pattern. The inhibition zone diameter of the extract decreased from 20.67 ± 0.58 mm to 12.50 ± 0.50 mm across a concentration range. This observed concentration-dependent activity is consistent with the general behavior of crude plant extracts, in which increasing concentration increases the amount of antimicrobial constituents available to interact with bacterial cells as Lawrence *et al.* (2016) reported that there is correlating increase in antimicrobial activity with increase in extract concentration.

The low MIC of the extract provides additional evidence of its activeness. The difference between MIC and MBC is also informative. MIC indicates the lowest concentration capable of inhibiting visible bacterial growth under the experimental conditions, whereas MBC represents the concentration capable of producing a bactericidal effect. The result therefore indicated that the extract inhibited bacterial growth at substantially lower concentrations than that required to produce the bactericidal endpoint. The MBC value of 1000 mg/ml for the extract suggests that the crude extract was more effective at inhibiting growth than at producing complete bacterial killing at lower concentrations.

Although *C. violaceum* infections in humans are uncommon, documented infections can be severe. Durán and Menck (2001)^[14] noted its capacity to cause septicemia and potentially fatal disease. The antibacterial effects demonstrated by *O. gratissimum* therefore provide preliminary evidence that locally available medicinal plants contain compounds capable of inhibiting the study organism. The findings are particularly relevant to natural-product research, where the plant is widely available and have a long history of traditional use.

Conclusion

This study investigated the antibacterial potential of *Ocimum gratissimum* leaf extract against *Chromobacterium violaceum*. The phytochemical analysis demonstrated that *O. gratissimum* extract contained several bioactive phytochemicals and showed concentration-dependent antibacterial activity against *C. violaceum* *in vitro*. Due to increasing antibiotic resistance in microorganisms and side effects of synthetic antibiotics, there is need for continuous research for medicinal herbs and plants that are potent, cheaper and readily available. *O. gratissimum* is readily available and is planted almost worldwide. The findings demonstrated that *O. gratissimum* extract possesses antibacterial activity against *C. violaceum* and can, therefore, be harnessed for production of drugs.

Recommendations

With the recent rise in antibiotic resistance and based on the findings of this study, further investigations should be done to determine the mechanisms by which the extract inhibit *C. violaceum* and harness it for effective phytotherapy

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