Phytochemical analysis and In-vitro screening of antimicrobial activities of *Ocimum gratissimum* on microorganisms isolated from wound infections.

Abstract

The in-vitro antimicrobial activities of crude ethanol and methanol extracts of the leaves of *Ocimum gratissimum* (scent leaf) was assessed on five clinical wound isolates (*Staphylococcus auerus*, *Escherichia coli*, *Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Candida albicans* using agar well diffusion method. The phytochemical constituents of this medicinal plant was carried out using standard methods. The Minimum inhibitory concentration (MIC) and minimum bactericidal/fungicidal concentration (MBC/MFC) of the plant extracts on the test isolates were determined by the agar dilution method. Ciprofloxacin and fluconazole (positive controls) were used in comparison with crude extract of *Ocimum gratissimum* leaves and also, Dimethyl sulfoxide (DMSO) was used as the negative control. The ethanolic extract of *Ocimum gratissimum* showed antibacterial activity with the mean inhibitory zone diameter of 3 -7mm against *Staphylococcus auerus,* 2 mm against *Escherichia coli,* 2 – 12 mm against *Klebsiella pneumonia,* 2 mm against *Pseudomonas aeruginosa. Candida albicans* was only found to be resistant to ethanol and methanol crude extracts of *Ocimum gratissimum* leaves. *Ocimum gratissimum* extracts showed the lower antimicrobial activity than the commercially available antibiotics (ciprofloxacin and fluconazole). The minimum Inhibitory Concentration and Minimum Bactericidal Concentration of the extracts on the test organisms also increased in the following order; methanol < ethanol. However, this plant extracts could be used as broad spectrum antibiotics in the treatment of wound infections since this leaf extracts has antimicrobial effects on bacterial pathogens. Secondary metabolites of this plant extracts could enhance rapid healing of wound infections.

Keywords: Antimicrobial activity, Phytochemical analysis, *Ocimum gratissimum,* Agar well assay, Ethanolic extract.

INTRODUCTION

A wound is an abrasion in the skin and the exposure of subcutaneous tissue following the loss of skin integrity which provide moist, warm and nutritious environment that is conducive for microbial colonization and proliferation [1]. Wounds can be classified as open or closed. They can further be classified as accidental, pathological or post-operative according to its nature [2]. Wound and other open lesions are liable to infection with a multiplicity of organism from the body surface or environment [2].

Infection occurs when one or more of the contaminants evade the cleaning effect of the host's defenses, replicate in large number, attack and harm the host and may best be described as colonization [3].

Endogenous infection or auto-infection is caused by organism that has been living a commensal existence in the patient's body [3].While exogenous infections are spread from person to person, this may occur after accident or intentional trauma of the skin or other tissue which is also called surgical or post-operative sepsis. Surgical site infection constitute a global health problem both health and human term [4]. Organisms commonly found in infected wounds include Gram positive cocci such as *S.aureus, Streptococcus spp,* Gram negative bacilli mostly *Enterobacter, E. coli, Proteus spp, P. aeruginosa* and *Klebsiella spp* [5].

With the increasing emergence of multiple antibiotics resistance, wound isolates are posing enormous public health concerns thus making the need for exploring possible alternatives a necessity[6]. Herbs and spices are very important and useful as therapeutic agent against many pathological infections [7]. Increasing multidrug resistance of pathogens has led the research for alternative compounds for treatment of infectious diseases [7].

Traditional treatment of circumcision wounds and chronic skin ulcers with locally prepared herbs and other natural occurring substances has been known for generations [8]. The plant is an erect small plumb with many barnacles usually not more than 1 m high [9]. These medicinal plants have been shown to be quite effective even where antibiotics treatments have failed [10].

Ocimum gratissimum also known as "alfavaca" is an aromatic medicinal plant belonging to the family *Lamiaceae*. It is an important herbal medicine found in the tropical and warm regions such as India and sub-Sahara Africa especially in Kenya and Nigeria [10]. In Nigeria, *Ocimum gratissimum is* called "Efinrin" in Yoruba; "Nchoanwu" or "Ahuji" in Igbo; "Aramogbo" in Edo and"Daidoya" in Hausa[11]

. It is of the family *Lamiaceae* , genus *Ocimum* and species *gratissimum* [12].

In traditional medicine, the leaves have been used as a general tonic and anti-diarrhea agent and for the treatment of conjunctivitis by instilling directly into the eyes; the leaf oil when mixed with alcohol is applied as a lotion for skin infections, and taken internally for bronchitis. The dried leaves are snuffed to alleviate headaches and fever among other uses [12]. In addition, despite the fact that the various extracts of *Ocimum gratissimum* have been tested *in vitro* and shown to be active against some bacteria and fungal isolates [13].

S. aureus forms a fairly large yellow colony on rich medium and is often hemolytic on blood agar. Staphylococci are facultative anaerobes that grow by aerobic respiration or by fermentation that yields principally lactic acid. The bacteria are catalase-positive and oxidase negative [3].

S. aureus is a major cause of hospital acquired (nosocomial) infection of surgical wounds and infections associated with indwelling medical devices [3].

Hospital strains of *S. aureus* are usually resistant to a variety of different antibiotics but few strains are resistant to all clinically useful antibiotics except vancomycin, and vancomycin resistant strains are increasingly-reported. Methicillin resistance is widespread and most methicillin-resistant strains are also multiple drug-resistant[14].

P.aeruginosa has become increasingly recognized as an emerging opportunistic pathogen of clinical relevance. Several different epidemiological studies track its occurrence as a nosocomial pathogen and indicate that antibiotic resistance is increasing in clinical isolates [14].

Furthermore, it is constantly reintroduced into the hospital environment on fruits, plants, vegetables, as well by visitors and patients transferred from other facilities. Spread occurs from patient to patient on the hands of hospital personnel, by direct patient contact with contaminated reservoirs, and by the ingestion of contaminated foods and water [15].

The spread of *P. aeruginosa* can best be controlled by observing proper isolation procedures, aseptic technique, and careful cleaning and monitoring of respirators, catheters, and other instruments [15].

P. aeruginosa is notorious for its resistance to antibiotics and is, therefore, a particularly dangerous and dreaded pathogen. Only a few antibiotics are effective against *P. aeruginosa*, including fluoroquinolones, gentamicin and imipenem, and even these antibiotics are not effective against all strains [15].

Theodor Escherich first described *E. coli* in 1885, as *Bacterium coli commune,* which he isolated from the feces of newborns. It was later renamed *Escherichia coli,* and for many years the bacterium was simply considered to be a commensal organism of the large intestine. Over 700 antigenic types (serotypes) of *E. coli* are recognized based on O, H, and K antigens[16].

Hence, analysis for pathogenic *E.coli* usually requires that the isolates first be identified as *E.coli* before testing for virulence markers[16].

Klebsiella is a genus of Enterobacteriaceae, a frequent cause of nosocomial pediatric infection. *Klebsiella* can cause infections of the urinary tract, lung, and central venous catheters in high-risk newborns and immunocompromised older children [17]. *Klebsiella* organisms were named for Edwin Klebs, the noted German bacteriologist [18].

K.pneumoniae accounts for less than 10 percent of hospitalized cases of pneumonia in adults [14]. *Klebsiella spp* now are in greatest evidence as opportunistic nosocomial pathogens of the urinary tract, respiratory tract, biliary tract, and bloodstream.

In one survey of the Centers for Disease Control and Prevention, the infection rate of nosocomial *K.pneumoniae* was 16.7 infections per 10,000 patients discharged [19]. Hand-carriage generally is regarded as the common mode of transmission [20].Environmental sources of *Klebsiella* spp. include contaminated blood-

pressure monitoring equipment [21], ventilator traps[20], ultrasound coupling gel [22], and dextrose solution [23].

Candida albicans cause the superficial fungal infections known as oral thrush, which occurs on the surface of the tongue and inside the mucus of the cheeks. It appears as white patches known as "plaques" which resemble milk curds [24].

It occurs most commonly in babies, particularly in the first few weeks of life
[24]. Outbreaks of thrush in older children may also be the result of an increased use of antibiotics and steroids, which disturbs the balance of microbes in the mouth.

Burnt patients are another population at high risk; the wound site is susceptible to colonization by opportunistic fungi such as *Candida*, but nowadays this is generally well managed and Candidiasis in burnt patients may originate in the gastrointestinal tract or from intravenous catheters [25].

This present study investigated the antimicrobial efficacy of *Ocimum gratissimum* extracts on *Staphylococcus auerus*, *Escherichia coli*, *Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Candida albicans* and in comparsion with ciprofloxacin and fluconazole in an attempt to further give further scientific backing to various tradomedical claims and uses of the leaves of *Ocimum gratissimum*

MATERIALS AND METHOD

Source and maintenance of test organisms

The wound isolates of *Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa and Candida albicans* were obtained from patient's samples in the surgical wards at NAUTH while Pure cultures of standard strains of *Staphylococcus aureus* (NCTC 6571) and *Escherichia coli* (NCTC 10418), (control organisms), were obtained from Department of Pharmaceutical Microbiology and Biotechnology, Nnamdi Azikiwe University, Awka, Nigeria.

Collection and identification of plant sample

Fresh leaves of *Ocimum gratissimum* was harvested from farms in Anambra State, Nigeria and identified in the Department of Botany, Nnamdi Azikiwe University, Awka by Mr Paulinus Ugwuoke.

Preparation of Extraction

Fresh leaves of *Ocimum gratissimum* was air dried after washing in distilled water at room temperature, grounded into fine powder with a mechanical grinder .Weighed 200 g of *Ocimum gratissimum* grounded powder and macerated in each 95% ethanol and methanol respectively for three days. After maceration, the

solution of the plant extracts were filtered through No. 1 What- man filter paper and the resulting solutions dried in a rotary evaporator at 60^{o}C. The dried extracts recovered were placed in sterilized screw-capped bottles and stored at 4^{o}C.

Phytochemical Analysis

The phytochemical analysis of methanol and ethanol extract of *Ocimum gratissimum* (scent leaf) was carried out using standard methods as described by [26].

Preparation of stock solutions

For the primary antimicrobial screening of the plant extracts, stock solutions were prepared by dissolving 400mg of the extracts in 2mL of DMSO (to make 200mg/mL). Also, in the determination of the minimum inhibitory concentrations of the plant extracts, stock solutions were prepared by dissolving 2000 mg/mL in 4mL of DMSO (to make 500mg/mL). These were stored in screw capped tubes at 4°C for further use.

In-vitro screening of antimicrobial activities of the plant leaf extracts.

The agar well diffusion assay method described by [27], was used to evaluate the antibacterial and antifungal activities of the crude extracts of *Ocimum gratissimum* against the test microorganisms. Dilutions of 100, 50, 25, 12.5, and 6.25mg/mL were prepared from the 200mg/mL stock solution of the plant extracts in a 2-fold dilution process. Twenty (20) mL of molten Mueller Hinton Agar (MHA) and Sabouraud Dextrose Agar (SDA) (for bacterial and fungal isolates respectively) were poured into sterile Petri dishes (90 mm) and allowed to set. Standardized concentrations (McFarland 0.5) of overnight cultures of test isolates were swabbed aseptically on the agar plates and holes (6mm) were made in the agar plates using a sterile metal cork-borer. Twenty (20µl) of the various dilutions of the plant extract and control were put in each hole under aseptic condition, kept at room temperature for one hour to allow the agents to diffuse into the agar medium and incubated accordingly. Ciprofloxacin (5µg/mL) and fluconazole (50µg/mL) were used as positive controls in the antibacterial and antifungal evaluations respectively; while DMSO was used as the negative control. The MHA plates were then incubated at 37^{o}C for 24 hours, and the SDA plates were incubated at room temperature (25-27^{o}C) for 2-3days. The inhibition zones diameters (IZDs) were measured and recorded. The size of the cork borer (6mm) was deducted from the values recorded for the IZDs to get the actual diameter.

This procedure was conducted in triplicate and the mean IZDs calculated and recorded.

Determination of Minimum Inhibitory Concentration (MIC) of the plant leaf extracts on test isolates

The Minimum inhibitory concentration (MIC) of the plant extracts on the test isolates were determined by the agar dilution method as described by [28].

The stock solutions (500mg/ml) were further diluted in a 2-fold serial dilution to obtain the following concentrations: 250, 125, 62.5, 31.25, 15.625, 7.8125, 3.91, 1.95, and0.98 mg/mL. Agar plates were prepared by pouring 4 mL of molten double strength MHA and SDA (for bacterial and fungal isolates respectively) into sterile Petri plates containing 1mL of the various dilutions of the extract making the final plate concentrations to become 100, 50, 25, 12.5, 6.25, 3.125, 1.5625, 0.78, 0.39, and 0.19 mg/mL.

The test isolates which were grown overnight in broth were adjusted to McFarland 0.5 standard and streaked onto the surface of the agar plates containing dilutions of the extract.

The MHA plates were then incubated at 37^{o}C for 24 hours and the SDA plates were incubated at room temperature (25-27^{o}C) for 2-3days, after which all plates were observed for growth.

The minimum dilution (concentration) of the extracts completely inhibiting the growth of each organism was taken as the MIC. This procedure was conducted in triplicate.

Determination of Minimum Bactericidal/Fungicidal Concentrations (MBCs/MFCs) of the plant leaf extracts on test isolates

The MBC/MFC of the plant extracts was derived by sub culturing portions of the agar from plates that showed no growth in the tests for determination of MICs. These agar portions were transferred respectively into plates containing freshly prepared MHA and SDA.

These plates were incubated at 25-27^{o}C for 2-3 days and were observed daily for growth. The absence of growth at the end of incubation period signifies total cell death.

The minimum concentration of the plant extracts that produces total cell death is taken as the MFC.

RESULTS

Qualitative phytochemical analysis detected the presence of tannins,reducing sugar,terpenoids,phenol,quinines,flavonoid and saponins in all plant extracts (Table 1).

The antibacterial effectiveness of the leaf extracts at concentrations of 200mg/ml,100mg/ml,25mg/ml and 6.25mg/ml as compared with the activity of ciprofloxacin was shown in Table 2. *Klebsiella pneumoniae* was only found to be susceptible to all the concentrations of crude ethanol extract of *Ocimum gratissimum* leaves with mean zone of inhibition ranging between 2- 12mm . Typed isolate of *Escherichia coli* and clinical isolate of *Candida alblicans* were found to be resistant to all the concentrations of crude ethanol extract of *Ocimum gratissimum* while typed and clinical isolates of *Escherichia coli, Klebsiella pneumoniae , Pseudomonas aeruginosa and Candida alblicans* were found to be resistant to all the concentrations of crude methanol extract of *Ocimum gratissimum* leaves (Table 3). However, the commerical antibiotics (Ciprofloxacin) showed greater antibacterial activity compared to its corresponding extract of ethanol and methanol.

Table 4 shows the MIC and MBC of the ethanol and methanol extract of plant. The ethanol extract showed the highest activity against clinical isolate of *K.pneumoniae,* then, *S.auerus* followed by *P.aeruginosa* and *E.coli.*

Table 1 : Phytochemical constituents of *Ocimum gratissimum* leaves using two solvents

S/N	**Phytochemical constituents**	**Methanol extract of *O.gratissimum***	**Ethanol extract of *O. gratissimum***
1.	Saponins	+	+
2.	Reducing sugar	+	+
3.	Terpenoids	+	+
4.	Tannins	+	+
5.	Phenols	+	+
6.	Quinones	+	+
7.	Glycosides	+	-

8.	Flavonoid	+	+
9.	Alkaloids	-	+

209

210 + : Detected

211 - : Not detected

212

213 **Table 2: Susceptibility Testing of Ethanol Extract of *Ocimum gratissimum* leaves showing the Inhibition**
214 **Zone Diameters (IZDs)(mm) produced by wound bacterial and yeast isolates**

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Concentrations of plant extract	200mg/ml	100mg/ml	50mg/ml	25mg/ml	12.5mg/ml	6.25mg/ml	Cipro floxacin	DMSO
Tested organisms	IZD(mm)	IZD(mm)	IZD(mm)	IZD(mm)	IZD(mm)	IZD(mm)	5ug/ml	
S.aureus (NCTC6571)	4	2	1	0	0	0	9	0
E.coli (NCTC10418)	0	0	0	0	0	0	12	0
S.auerus	7	5	3	0	0	0	12	0
E.coli	2	0	0	0	0	0	22	0
K.pneumoniae	12	10	8	6	4	2	10	0
P.aeruginosa	2	0	0	0	0	0	12	0
							Fluco	

							nazole 50ug/ml	
C.albicans	0	0	0	0	0	0	24	0

0: Resistant

Table 3: Susceptibility Testing of Methanol Extract of *Ocimum gratissimum* leaves showing the Inhibition Zone Diameters (IZDs)(mm) produced by wound bacterial and yeast isolates

Concentrations of plant extract	200mg/ml	100mg/ml	50mg/ml	25mg/ml	12.5mg/ml	6.25mg/ml	Cipro floxacin 5ug/ml	DMSO
Tested organisms	IZD(mm)	IZD(mm)	IZD(mm)	IZD(mm)	IZD(mm)	IZD(mm)		
S.aureus (NCTC6571)	4	2	1	0	0	0	9	0
E.coli (NCTC10418)	0	0	0	0	0	0	12	0
S.auerus	8	6	4	2	1	0	12	0
E.coli	0	0	0	0	0	0	22	0
K.pneumoniae	0	0	0	0	0	0	10	0
P.aeruginosa	0	0	0	0	0	0	12	0
							Fluco nazole	

							50ug/ml	
C.albicans	0	0	0	0	0	0	24	0

0 : Resistant

Table 4 :MIC and MBC of the extracts against tested organisms.

Isolates	Ethanol		Methanol	
	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)
S.auerus (NCTC6571)	50	50	50	50
E.coli (NCTC10418)	-	-	-	-
S.auerus	25	25	12.5	25
E.coli	200	200	-	25
K.pneumoniae	3.125	3.125	-	200
P.aeruginosa	200	200	-	-
C.albicans	-	-	-	-

- No Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal/Fungicidal Concentration (MBC/MFC)

DISCUSSION

Since this leaf extracts has been found to be susceptible to the bacterial isolates, there is likely that this could be used as a broad spectrum antibiotics in the treatment of wound infections. Secondary metabolites of this plant extracts contain antioxidants which enhance rapid healing of wound infections. Qualitative phytochemical analysis detected the presence of tannins, reducing sugar,terpenoids,phenol,quinines,flavonoid and saponins in all plant extracts .The antibacterial effectiveness of the leaf extracts at concentrations of 200mg/ml,100mg/ml,25mg/ml and 6.25mg/ml as compared with the activity of ciprofloxacin . *Klebsiella pneumoniae* was only found to be susceptible to all the concentrations of crude ethanol extract of *Ocimum gratissimum* leaves with mean zone of inhibition ranging between 2- 12mm . Typed isolate of *Escherichia coli* and clinical isolate of *Candida alblicans* were found to be resistant to all the concentrations of crude ethanol extract of *Ocimum gratissimum* while typed and clinical isolates of *Escherichia coli, Klebsiella pneumoniae , Pseudomonas aeruginosa* and *Candida alblicans* were found to be resistant to all the concentrations of crude

methanol extract of *Ocimum gratissimum* leaves. This present study was not in line with the work of [13],who shown the various activities of *Ocimum gratissimum* extract tested *in vitro* against some bacteria and fungal isolate.

However, the commerical antibiotics (Ciprofloxacin) showed greater antibacterial activity compared to its corresponding extract of ethanol and methanol. This is possibly due to the failure of the active ingredient to dissolve in it and all the sensitive extracts were more at higher concentrations than lower concentration. Also, the comparsion of the activity of the plant extract with conventional antibiotics, such as ciprofloxacin and fluconazole confirmed reports by other workers [29], that constitutional antibiotics are more active than plant extracts. The ethanol extract showed the highest activity against clinical isolate of *K.pneumoniae,* then, *S.auerus* followed by *P.aeruginosa* and *E.coli.* The ethanolic extract was both bacteriostatic and bacteriocidal at a concentration of 3.125mg/ml, 25mg/ml, 50mg/ml and 200mg/ml on the clinical isolate of *K.pneumoniae,* clincal isolate of *S.auerus ,* typed isolate of *S.auerus,* clinical isolates of *P.aeruginosa* and *E.coli* respectively while the methanolic extract was both bacteriostatic and bacteriocidal at a concentration of 50mg/ml on the typed isolate of *S.auerus*.

Conclusion

Ethanolic extract of *Ocimum gratissimum* was observed to be more susceptible to *K.pneumoniae* at all concentrations, thus showing higher antibacterial activity than the methanolic extract. *Candida alblicans* was found to be resistant at any concentrations of crude extract of *Ocimum gratissimum* leaves. Consequently, failure of some of the extract to exert antimicrobial effect on the test organism is not enough to conclude that the leaves do not contain substances that can exert antimicrobial activity against the test organism because the potency of extract depends on method used to obtain the extract. However, the higher the concentration of the antimicrobial agents in the extracts, the increase the effectiveness of the extracts on the organisms[30].

Further attention and research to identify the active components responsible for the plant antifungal activity should also be carried out.

Study should be done on extensive investigation, isolation and purification of active phyto constituents with broad spectrum of antimicrobial activity.

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