AFRICAN JOURNAL OF CLINICAL AND EXPERIMENTAL MICROBIOLOGY SEPTEMBER 2004 ISSN 1595-689X VOL 5 NO 3
AJCEM/2003040/2436

EFFECT OF ESSENTIAL LEAF OIL OF *OCIMUM GRATISSIMUM* ON CLINICAL ISOLATES OF *PSEUDOMONAS AERUGINOSA*

Iwalokun, B. A., Owuh, I. G., Ronke, S. A.

Department of Biochemistry, Lagos State University, PMB 1087, Apapa, Lagos, Nigeria

Correspondence to: B. A. Iwalokun (bamwal@yahoo.com)

***Ocimum gratissimum* leaf oil, which has been reported to possess *in vitro* and *in vivo* efficacy against enteric bacteria was tested against forty six clinical isolates of Pseudomonas aeruginosa in Lagos, Nigeria. The effect of the essential oil (EO) on pyocyanin production among these strains was also investigated. Agar well diffusion assay revealed susceptibility in 40 (87%) of the 46 tested strains with inhibition zone diameter (20-36 mm) comparable with the effect of tobramycin. Of the 40 susceptible isolates, 34 strains were quantitatively demonstrated to show susceptibility when further tested with the essential oil in broth and on agar yielding MIC and MBC values of 36 – 54 mg/ml and 42 – 66 mg/ml respectively. The values were higher than the 12 mg/ml (MIC) and 24 mg/ml (MBC) observed in *Escherichia coli* ATCC 25922. Compared with the control, the essential oil was found to reduce pyocyanins production significantly ($p < 0.01$) at 15 mg/ml (30.1 – 30.5 vs 259.2 – 276. 7 µg/ml) and 75 mg/ml (2.5 – 3.5 vs 259.2 – 276.7 µg/ml) in both sensitive and resistant strains, suggesting that *Ocimum gratissimum* leaf oil may inhibit expression of virulence factors and progression of *Pseudomonas* infections caused by the tested strains.**

Key words: *Ocimum gratissimum* leaf oil, *Pseudomonas aeruginosa*, Nigeria

INTRODUCTION

On a global scale, *Pseudomonas aeruginosa* is responsible for 16% of all nosocomial infections and 5% of all community-acquired infections (1). In Nigeria, hospital acquired pseudomonal infections are common among patients on in-dwelling devices and those with chronic discharging ears and bronchopulmonary disorders (2). These diseases often lead to complications such as septicaemia with eventual death (3). The large numbers of diseases caused by *Pseudomonas aeruginosa* as an opportunistic pathogen is premised on many factors, which include multi-drug resistance and production of virulence factors such as pyocyanin.

Infections caused by multi-drug resistant strains of *Pseudomonas aeruginosa* have severally been reported in Nigeria (4, 5, 6). The gross resistance of *Pseudomonas aeruginosa* to antibiotics has paved way for an extensive exploration of plants of folkloric medicine for phytochemicals against this organism throughout the globe (7, 8, 9).

Plants' parts such as the seeds of *Moringa oleifera* and *Nigella sativa* (10, 11), roots of *Diospyros mespiliformis* (12), fruits of *Juniperus oxycedrus* (13) and leaves of *Synclisia scariba* and *Bryophyllum pinnatum* (14, 15) have been found to possess antibacterial activity against *Pseudomonas aeruginosa*. However, the use of these plants for complementary therapies is limited by their narrow geographical spread and inadequate folklore medicinal belief mostly in unfamiliar rural communities.

Ocimum gratissimum is one of the medicinal plants that are widely used in Nigeria and acclaimed to have a large clinical coverage of diseases in folk medicine (16). The extracts of the plants have severally been demonstrated to possess bacteriologic and clinical efficacy against infections due to *Enterobacteriaceae* (17, 18). *In vitro*, we have found the essential oil of *Ocimum gratissimum* inhibitory to extracellular protease elicited as a virulent factor by *Shigellae* (article in-press) and a study by Fakae *et al* (19) demonstrated the

ability of the oil to inhibit glutathione – S - transferase activity in helminthic infections. Elsewhere the anti-pseudomonal effect of basil oil chemotypes on *Pseudomonas spp* has been reported (20) amidst contradictions from a related study (21).

In Nigeria, there are no documented studies demonstrating the interactions between *Ocimum gratissimum* leaf oil and *Pseudomonas aeruginosa*. We hypothesize that knowledge of the efficacy of *Ocimum gratissimum* leaf oil on *Pseudomonas aeruginosa* may improve and cheapen intervention measures against Pseudomonal infections. Hence, the present study was conducted to investigate the effect of *Ocimum gratissimum* leaf on growth and pyocyanin production among clinical strains of *Pseudomonas aeruginosa* isolated in Lagos, Nigeria.

MATERIALS AND METHODS

Ocimum gratissimum

Fresh leaves of *Ocimum gratissimum* (*Labiatae*) attached to their stems with leaves were authenticated at the Forestry Research Institute (FRIN), Ibadan, Nigeria. After authentication, the specimen was given a voucher number FHI 106506 and deposited in the forestry herbarium.

Essential leaf oil extraction

Three hundred grammes of *Ocimum gratissimum* leaves cut into pieces were steam distillated with the aid of a distillation apparatus (Borhringer, Germany). The resulting distillate was fractionated with petroleum ether (40–60°C) and extracted oil was dried on anhydrous sodium sulphate (Sigma, USA). Petroleum ether was removed by evaporation in vacuo at 50°C using a rotary evaporator. The amount of oil obtained as a percentage the quantity of leaf distillated was determined and recorded. The oil extract was reconstituted by dissolution in 10ml of 2% Tween-80 and filtered sterilized by passage through a 0.45µm filtration apparatus (21).

Bacterial isolates

Pure stock cultures of 46 clinical isolates of *Pseudomonas aeruginosa* maintained on nutrient agar slants within 4 weeks of isolation at 4°C and obtained from the Genetics units of the Nigerian Institute for Medical Research (NIMR), Lagos were used in this study. These isolates were identified and purified by culturing samples (blood, urine, stool, wound exudates and ear discharge) first on MacConkey and then on *Pseudomonas* agar medium supplemented with cetrimide (200 µg/ml) and sodium nalidixate (15 µg/ml). *Escherichia coli* ATCC 25922 was employed to validate outcomes of antibacterial susceptibility testing. All the bacterial strains were obtained from the Microbiology and Genetic division of the Nigerian Institute of Medical Research (NIMR), Lagos, Nigeria.

Antibacterial susceptibility testing

The sensitivity of the selected *Pseudomonas aeruginosa* strains to *Ocimum gratissimum* essential oil (EO) was determined by agar-well diffusion method. Cultures were grown in nutrient broth to late exponential phase (12 – 18 hours). The resulting turbidity was adjusted to 0.5 McFarland standard (1 x 10^8CFU/ml) using phosphate buffered saline (pH 7.4). 10 µL of standardized inoculum (10^5CFU/plate) was then used to streak Mueller Hinton agar (20 ml). Four 6 mm holes placed 4 cm apart were immediately bored on each inoculated plate using heat sterilized capillary tubes. After air-drying, the holes were seeded with

100 µL each of EO at 5000 µg/hole and 2% Tween-80 diluent for test and control. The plates were further mounted with a standard antibiotic tobramycin disk (20 µg) from Oxoid, UK, to serve as a negative control. Control plates inoculated with *Escherichia coli* ATCC 25922 were also prepared in parallel with the test experiment to validate sensitivity. All the plates were incubated aerobically at 37°C for 24 hours. Zones of inhibition were measured in millimeters (mm) and recorded.

Minimum inhibitory concentration (MIC)

The inhibitory effect of EO on each of the 46 *Pseudomonas aeruginosa* isolates was determined based on dilution in broth according to Ilori *et al* (17) with a little modification. A concentration range of 0 – 72 mg/ml of EO was prepared using Mueller Hinton broth as the diluent. The susceptibility test broth tubes were then made up to 4 ml by adding 100 µL of culture (1 x 10^6 CFU/ml) of the tested strains. EO negative tubes and EO tubes inoculated with *Escherichia coli* ATCC 25922 served as controls. The tubes were plugged with sterile cotton wool and incubated aerobically at 37°C for 24 hours. The MICs were regarded as the lowest concentration of EO at which no visible growth or turbidity occurred. Turbidity of cultures was monitored spectrophotometrically at 620 nm against sterile nutrient broth.

Minimum bactericidal concentration (MBC)

Here, 10µL sample taken from MIC tubes without bacterial growth or turbidity was inoculated onto Mueller Hinton agar and plates were subsequently incubated accordingly for 24 hour. MBCs were defined as the lowest concentration of EO that yielded no colonies of strains tested after incubation.

O.gratissimum effect on pyocyanin production

Each *Pseudomonas aeruginosa* strain was grown under iron limiting condition in tris-minimal succinate medium containing 25 µM ethylenediaminedi-o-hydroxyphenylacetic acid (EDDA), with and without 20 or 72 mg/ml EO. Culture negative broths were used as control. After 24 hours incubation at 37°C, pyocyanin production was estimated as described by Rogers (22). In brief, pyocyanin producing strains were grown aerobically overnight at 37°C with shaking (120 rpm) in tris-minimal succinate solution without iron and glucose but containing $MgCl_2$ (500µM), $CaCl_2$ (100µM) and methionine (700 µM). Cultures were then centrifuged at 4000 rpm for 10 minutes and supernatant acidified with ethyl acetate in volume 5:2 ratio. The acidified pyocin fraction was then concentrated under reduced pressure using a rotary evaporator at 50°C. The crude pyocin preparation obtained was then dissolved in 400µL sterile water, sterilized by passage through a 0.45 µM filtration unit and the yield measured in µg/ml. A non-pyocin strain of *Pseudomonas putida* PUC 34 was used as control.

Statistical analysis

Pyocyanin production among the *Pseudomonas aeruginosa* strains tested measured in µg/ml was expressed as means ± standard deviation. Differences between mean values were further subjected to student's t-test with level of significance investigated at 99% confidence limits. $P < 0.01$ was considered significant.

RESULTS

The data obtained following the extraction of essential oil (EO) from *Ocimum gratissimum* leaves and effects on growth of 46 clinical isolates of *Pseudomonas aeruginosa* are summarized. Table 1 revealed the production of 720 mg of essential oil equivalent to 0.24% oil yield from 300g of *Ocimum gratissimum* leaves following steam distillation with petroleum ether. Forty of the 46 tested *Pseudomonas aeruginosa* strains showed susceptibility to the essential oil and produced an inhibition zone (18 – 34 mm) that was comparable to the bactericidal effects of tobramycin (20 – 32 mm). All the tested strains formed colonies resulting in the absence of inhibition zones when grown on 2% Tween-80 agar (plates not shown). Results in table 2 indicated that *Ocimum gratissimum* EO at 36 – 54 mg/ml and 42 – 66 mg/ml concentrations inhibited the growth of 36 of the 40 sensitive strains in broth and on agar *in vitro*. Minimum inhibitory concentrations (MICs) of the remaining 6 sensitive strains were observed at ≥ 72 mg/ml EO. MIC of 12 mg/ml and MBC of 24 mg/ml EO were obtained following interactions with the control strain, *Escherichia.coli* ATCC 25922.

A decrease in pyocyanin production was also observed in the order of 259 ± 86.7, 30.1 ± 10.2 and 2.5 ± 1.5 µg/ml as a result of the growth of the *Pseudomonas aeruginosa* sensitive strains in media containing tris succinate and EDDA, 15mg/ml EO and EDDA and 75mg/ml EO and EDDA. The disparity in pyocin yield as a result of growth in the presence of EO was found to be significant ($p < 0.01$) (Table 3).

The data presented in Table 4 also showed a significant decrease ($p < 0.01$) in pyocyanin production (276.7 ± 23.1 vs 3.5 – 30.5 ± (1.0 – 2.3) µg/ml) among the EO resistant strains when cultured in the three categories of media used.

Table 1: Essential oil yield and antibacterial susceptibility testing of *Pseudomonas aeruginosa* strains with *Ocimum gratissimum* essential oil (EO) by agar well diffusion method

	O. gratissimum EO	Tobramycin	Tween-80
Organism tested			
P. aeruginosa (N = 46).	18 – 34[a]	20 – 32[b]	0*
E. coli (ATCC2592) (N = 4).	22 – 30[b]	20 – 36[b]	0*

- Essential oil yield = 720mg per 300g leaf.
- Percentage oil yield = 0.24%

N = number of strains tested or determinations; a = 40 *P. aeruginosa* strains tested were susceptible to EO; b = all the strains tested were susceptible to EO or tobramycin. Figures were a range of inhibition zone diameters measured in millimeters (mm). * = All the tested strains formed colonies on 2% Tween-80 control agar plates.

Table 2: Determination of MICs and MBCs of *Ocimum gratissimum* EO on the susceptible *Pseudomonas aeruginosa* strains

	Pseudomonas aeruginosa		*Escherichia coli* ATCC25922
	N = 34	N = 6	
Parameters			
MIC (mg/ml)	36 – 54	≥ 72	12
MBC (mg/ml)	42 – 66	ND	24

ND = growth inhibition not detected within the EO concentration tested.
E. coli ATCC 25922 inoculations was done in quadruplicate.

Table 3: Effects of *Ocimum gratissimum* essential oil on pyocyanin production in susceptible *Pseudomonas aeruginosa* strains

Tests	Pyocyanin production (µg/ml ± SD)
	N = 40
15mg/ml EO + EDDA	30.1 ± 10.2@
72mg/ml + EDDA	2.5 ± 1.5@
Tris-succinate + EDDA	259.2 ± 86.7

N = number of strains tested; @ = Significantly reduced pyocyanin yield at 99% confidence limits

Table 4: Effects of *Ocimum gratissimum* essential oil on pyocyanin production in resistant *Pseudomonas aeruginosa strains*

Test	Pyocyanin production (µg/ml ± SD)
	N = 6
15mg/ml EO + EDDA	30.5 ± 2.3@
72mg/ml + EDDA	3.5 ± 1.0@
Tris-succinate + EDDA	276.7 ± 23.1

N = number of strains tested; @ = Significantly reduced pyocyanin yield at 99% confidence limits

DISCUSSION

Ocimum gratissimum has been credited to have tremendous clinical benefits in folk medicine prior to the exploration of its chemotypic potentials and the propagation of modern medicine (16). The present study has an intention to expand the scientific basis of *Ocimum gratissimum* essential oil as an antibacterial agent. The 0.24% oil obtained in this study is comparable with yields reported in previous works (21, 23). The essential oil was further found to inhibit the growth of 40 of 46 tested strains on agar medium with 18 – 34 mm zone of inhibition. The MIC and MBCs values of 36 – 54 mg/ml and 42 – 66 mg/ml observed among the susceptible strains are contrary to the report of Nakamura *et al* (21). These workers found the eugenol containing EO un-inhibitory to *Pseudomonas aeruginosa* at 24 mg/ml. Other causes of disparity may include the chemotype variation common to basil plants including *Ocimum gratissimum* (24), experimental design and the strains of *Pseudomonas aeruginosa* tested. The latter could result from clonal variation in the acquisition of R-plasmids and other virulence determinants as demonstrated in the work of Mucha and Farrand (25). However, our result is comparable to the efficacy reported for the essential oil of *Cinnomonium osmophloeum* (26). It also aligns with the microbiological safety

appraisals given to the plant essential oils of Australian origin (27, 28).

Furthermore, in support of our result is the finding of Orafidiya *et al* (29) in which liquid and semi solid formulations of *Ocimum gratissimum* EO were found efficacious as a topical agent in the treatment of boil, pimples and wounds. Strains of *Pseudomonas aeruginosa* have been found responsible for foot puncture wound infections in children (30) and many studies have implicated *Pseudomonas aeruginosa* as among the aetiologic agents of skin infections (31). Keita *et al* (32) also reported concentrations of *Ocimum gratissimum* EO greater than 60 mg/ml in the control of *Callosobruchus maculatus* while in another study, concentrations as high as 62 mg/ml were found inhibitory against enteric bacteria (17).

The reduction in pyocyanin production among the strains tested irrespective of the susceptibility outcomes further demonstrates the spectrum of activity inherent in *Ocimum gratissimum* EO. Pyocyanins confer virulence to pseudomonas in a manner that employs cell-to-cell signaling system through which cell multiplication and further production of virulence determinants are ensured (33). Hence, inhibition of pyocyanin production may prevent the development of these processes. This observation further mimics the finding of Tateda *et al* (34) in which sub inhibitory concentrations of macrolides inhibit protein synthesis and suppress virulence factors in *Pseudomonas aeruginosa.*

The realization of *Ocimum gratissimum* essential oil as an antipseudomonal agent will be highly rewarding especially in rural areas that are far from adequate health facilities. The oil can easily be applied as drops and topical agent in the management of pseudomonas associated ear, eye and wound infections in these circumstances.

ACKNOWLEDGEMENT

The authors wish to thank the entire staff of the Genetics Laboratory of the Nigerian Institute for Medical Research (NIMR) and Mr. Adeleke from Pharmacognosy Department, College of Medicine University of Lagos for their technical assistance.

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