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In Vitro Susceptibility of *Cinnamomum verum* Against Multidrug-Resistant *Pseudomonas aeruginosa* by Broth Microdilution

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ABSTRACT

Background: Multidrug-resistant (MDR) *Pseudomonas aeruginosa* leaves clinicians with few reliable treatment options, which keeps interest in plant-derived antimicrobials alive. Data on *Cinnamomum verum* (true cinnamon) against well-characterised clinical MDR isolates, tested by a standardised dilution method, are limited.

Objective: To determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of *C. verum* against clinical MDR *P. aeruginosa* by broth microdilution, and to test whether activity depends on the isolates' antibiotic resistance profile.

Methods: We tested 145 clinical isolates of MDR *P. aeruginosa* from a tertiary care hospital. Antibiotic susceptibility was determined against 13 agents. *C. verum* MIC was measured by broth microdilution in two-fold serial dilutions, and MBC by subculture of clear wells. MIC and MBC were compared across resistance phenotypes and specimen types with non-parametric tests.

Results: All isolates were non-susceptible in at least three antibiotic categories; 111 (76.6%) were non-susceptible in five or more, and all were resistant to at least one carbapenem. *C. verum* MICs ranged from 0.078 to 5 mg/mL (MIC50 0.312 mg/mL, MIC90 2.5 mg/mL, geometric mean 0.376 mg/mL); 119 isolates (82.1%) were inhibited at 0.312 mg/mL or less. MBCs ranged from 0.156 to 10 mg/mL (MBC50 0.625 mg/mL, MBC90 5 mg/mL). The MBC/MIC ratio was 4 or less for every isolate (2 in 132; 4 in 13), indicating bactericidal activity. MIC did not differ by resistance to any of the 13 antibiotics, including carbapenems and colistin (all $P > 0.05$), did not correlate with the number of antibiotics resisted (Spearman $\rho = -0.10$, $P = 0.23$), and did not differ by specimen type ($P = 0.96$).

Conclusion: *C. verum* showed uniform, bactericidal in vitro activity against MDR *P. aeruginosa* at 0.312–0.625 mg/mL for most isolates, independent of the antibiotic resistance profile. The concentrations are modest for a crude preparation, so toxicity, composition and combination studies must follow before any clinical claim.

Keywords: *Cinnamomum verum*; *Pseudomonas aeruginosa*; multidrug resistance; broth microdilution; minimum inhibitory concentration; minimum bactericidal concentration; antimicrobial resistance

INTRODUCTION

Pseudomonas aeruginosa is a leading cause of hospital-acquired pneumonia, bloodstream, urinary tract, wound and device-associated infections, and it is notorious for resisting antibiotics [1,2]. Its outer membrane is poorly permeable, it carries multidrug efflux pumps and chromosomal AmpC β-lactamase, and it readily acquires carbapenemases, aminoglycoside-

modifying enzymes and target mutations [2–4]. Carbapenem-resistant *P. aeruginosa* is classed by the World Health Organization as a high-priority pathogen for which new treatments are needed [5,6]. Global estimates attribute a large share of the burden of bacterial antimicrobial resistance to such organisms [7]. For isolates resistant to most β-lactams, fluoroquinolones and carbapenems, colistin and newer β-lactam/β-lactamase inhibitor combinations are often the only remaining options, and these are costly, toxic or not widely available.

This has renewed interest in plant-derived antimicrobials, including spice essential oils and extracts [8]. *Cinnamomum verum* J. Presl (syn. *C. zeylanicum* Blume), or true cinnamon, is widely used as a food and traditional medicine. Its bark contains cinnamaldehyde as the main bioactive constituent, with eugenol, linalool and other phenolics in smaller amounts [9–11]. Cinnamaldehyde damages bacterial membranes, inhibits cell division and enzyme activity, and interferes with quorum sensing, which are mechanisms unrelated to those of conventional antibiotics [12–14]. Cinnamon preparations have been reported to inhibit *P. aeruginosa*, including multidrug-resistant clinical isolates, and to act synergistically with some antibiotics [15,16]. *C. verum* also contains far less coumarin than *C. cassia*, a point that matters for safety [17].

Two gaps limit the value of the existing reports. First, many studies used agar diffusion, which depends on diffusion of the test material and gives zones of inhibition that cannot be compared between laboratories or converted into a concentration [18,19]. Quantitative dilution methods, especially broth microdilution, are the accepted reference for MIC determination, and are recommended for natural products as well [18,20]. Second, most studies tested small numbers of isolates or reference strains, so they say little about the spread of MIC values in a resistant clinical population or about whether activity depends on the resistance phenotype.

Accordingly, we determined the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of *Cinnamomum verum* against 145 clinical multidrug-resistant (MDR) *Pseudomonas aeruginosa* isolates using the broth microdilution method. Furthermore, we investigated the potential association between susceptibility to *C. verum* and resistance profiles to 13 antibiotics, the cumulative number of antibiotic classes to which the isolates exhibited resistance, and the clinical specimen type from which each isolate was recovered.

MATERIALS AND METHODS

Study design and setting

This was a cross-sectional in vitro study done in the Department of Microbiology, Index Medical College Hospital and Research Centre, Malwanchal University, Indore, Madhya Pradesh, India. over a period of 2 years. The study followed CLSI broth dilution methodology [20].

Bacterial isolates

We included 145 clinical isolates of *P. aeruginosa* from patients in medical, surgical and paediatric wards and intensive care units. Isolates were identified by Standard Microbiological Techniques**.** Age, sex, ward and specimen type were recorded from laboratory requisition forms. The study received clearance from the Institutional Ethics Committee of Index Medical College Hospital and Research Center, affiliated with Malwanchal University, under approval number MU/Research/EC/Ph.D./2023/300.

Antibiotic susceptibility testing and resistance categories

Susceptibility was tested by Kirby–Bauer disk diffusion method and interpreted by CLSI breakpoints M100 35edition. [21]. The panel had 13 agents: ceftazidime (CAZ), cefepime (CPM), piperacillin–tazobactam (PIT), tobramycin (TOB), amikacin (AK), ciprofloxacin (CIP), levofloxacin (LE), imipenem (IMP), meropenem (MRP), aztreonam (AT), colistin (CL), ceftazidime–avibactam (CAZ-AV) and ceftazidime–clavulanic acid (CAC);. Results were recorded as susceptible (S) or resistant (R). Colistin sensitivity was done using colistin screen agar.



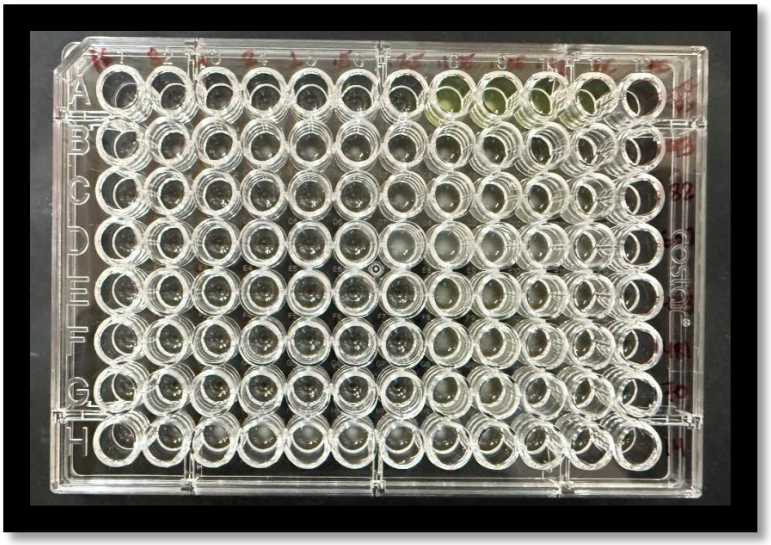
Isolates were classed as MDR when non-susceptible to at least one agent in three or more antimicrobial categories [22]. Seven categories were applicable: Antipseudomonal cephalosporins (CAZ, CPM), Penicillin/β-lactamase inhibitor (PIT), Aminoglycosides (TOB, AK), Fluoroquinolones (CIP, LE), Carbapenems (IMP, MRP), Monobactam (AT) and Polymyxin (CL). Carbapenem resistance was defined as resistance to imipenem or meropenem. Difficult-to-treat resistance was defined as resistance to all of piperacillin–tazobactam, ceftazidime, cefepime, aztreonam, meropenem, imipenem, ciprofloxacin and levofloxacin.

Test material

C. verum bark was shade-dried, powdered, extracted with 80 % ethanol, and reconstituted in dimethyl sulfoxide(DMSO).The material was diluted to a stock of 10 mg/mL and sterilised by 0.22 µm filtration. The final solvent concentration in wells was ranging from 0.078 mg/ml to 10 mg/ml, and a solvent control was included.

Broth microdilution for MIC

MIC was determined in sterile 96-well microtitre plates by broth microdilution as described in CLSI M07 [20] and by Wiegand et al. [18]. Two-fold serial dilutions of the test material were made in cation-adjusted Mueller–Hinton broth to cover 0.078 mg/ml to 10 mg/mL. Colonies from an overnight non-selective agar plate were suspended in saline to 0.5 McFarland (about 1.5×10^8 CFU/mL) and diluted so that each well held about 5×10^5 CFU/mL. Every plate had a growth control (broth and inoculum), a sterility control (broth only) and the solvent control. *P. aeruginosa* ATCC 27853 was tested with each batch. Plates were incubated at 35 ± 2 °C for 16–20 h. The MIC was the lowest concentration with no visible growth, read with **turbidity** [23]. Tests were run in **triplicate**.



Determination of MBC

From each well at and above the MIC, 10 µL was subcultured onto **Muller–Hinton agar** and incubated at 35 ± 2 °C for 24 h. The MBC was the lowest concentration giving no growth (a 99.9% or greater reduction of the initial inoculum). An MBC/MIC ratio of 4 or less was taken as bactericidal and a ratio above 4 as bacteriostatic [24].

Statistical analysis

MICs and MBCs are reported as ranges, MIC50 and MIC90 (the concentrations inhibiting 50% and 90% of isolates), MBC50 and MBC90, and geometric means. Because dilution data are discrete and skewed, groups were compared with non-parametric tests: Mann–Whitney U for MIC in resistant versus susceptible isolates of each antibiotic, Kruskal–Wallis across specimen groups, and Spearman rank correlation between MIC and the number of antibiotics or categories resisted. Tests were two-sided with $\alpha = 0.05$; for the 13 antibiotic comparisons a Bonferroni threshold of 0.0038 is also given. Analyses used Python 3 with SciPy.

RESULTS

Isolate characteristics

The 145 isolates came from 84 men (57.9%) and 61 women (42.1%). Among the 141 patients with age recorded in years, the median age was 51 years (interquartile range 35–63); four further patients were infants under 1 year. Isolates were most often from pus, wound and other swabs (39.3%), followed by respiratory (22.8%) and urine (21.4%) specimens, and 13 (9.0%) were from blood (Table 1). Surgery accounted for 53 isolates (36.6%).

Table 1. Characteristics of 145 MDR *P. aeruginosa* isolates

Characteristic	n (%)
Male sex	84 (57.9)
Age <18 years	17 (11.7)
Age 18–59 years	79 (54.5)
Age ≥60 years	49 (33.8)
Specimen: pus, wound or other swab	57 (39.3)
Specimen: respiratory (sputum, endotracheal tube, tracheal aspirate, BAL)	33 (22.8)
Specimen: urine	31 (21.4)
Specimen: blood	13 (9.0)
Specimen: ear swab	5 (3.4)
Specimen: other sterile site or device tip	6 (4.1)
Ward: Surgery	53 (36.6)
Ward: ENT	14 (9.7)
Ward: General Medicine	14 (9.7)
Ward: Urology	13 (9.0)
Ward: Pulmonary Medicine	12 (8.3)
Ward: Orthopaedics	9 (6.2)
Ward: Obstetrics and Gynaecology	9 (6.2)
Ward: Paediatrics	5 (3.4)
Ward: all others	16 (11.0)

Antibiotic resistance profile

Resistance was highest to levofloxacin (83.4%), ciprofloxacin (81.4%) and aztreonam (80.0%), and lowest to colistin (13.8%) and tobramycin (27.6%) (Table 2).

Every isolate was resistant to at least one carbapenem: 59 (40.7%) to both, 46 (31.7%) to imipenem only and 40 (27.6%) to meropenem only. Twenty isolates (13.8%) were colistin-resistant, and 13 of these were also resistant to both carbapenems. Fourteen isolates (9.7%) met the definition of difficult-to-treat resistance.

Table 2. Antibiotic resistance of 145 isolates and *C. verum* MIC by resistance status

Antibiotic	Resistant, n (%)	Susceptible, n (%)	Median MIC, resistant vs susceptible (mg/mL)	*P* (Mann–Whitney)
Ceftazidime	112 (77.2)	33 (22.8)	0.312 vs 0.312	0.29
Cefepime	77 (53.1)	68 (46.9)	0.312 vs 0.312	0.31
Piperacillin–tazobactam	101 (69.7)	44 (30.3)	0.312 vs 0.312	0.67
Tobramycin	40 (27.6)	105 (72.4)	0.312 vs 0.312	0.41
Amikacin	62 (42.8)	83 (57.2)	0.312 vs 0.312	0.90
Ciprofloxacin	118 (81.4)	27 (18.6)	0.312 vs 0.312	0.98
Levofloxacin	121 (83.4)	24 (16.6)	0.312 vs 0.312	0.48
Imipenem	105 (72.4)	40 (27.6)	0.312 vs 0.312	0.56
Meropenem	99 (68.3)	46 (31.7)	0.312 vs 0.312	0.61
Aztreonam	116 (80.0)	29 (20.0)	0.312 vs 0.312	0.60
Colistin	20 (13.8)	125 (86.2)	0.312 vs 0.312	0.14
Ceftazidime–avibactam	58 (40.0)	87 (60.0)	0.312 vs 0.312	0.10
Ceftazidime–clavulanic acid	87 (60.0)	58 (40.0)	0.312 vs 0.312	0.38

MIC and MBC of *C. verum*

C. verum inhibited all 145 isolates. MICs ranged from 0.078 to 5 mg/mL, with MIC50 0.312 mg/mL, MIC90 2.5 mg/mL and a geometric mean of 0.376 mg/mL (Table 3). The distribution had one clear mode: 104 isolates (71.7%) had an MIC of

0.312 mg/mL, and 119 (82.1%) were inhibited at 0.312 mg/mL or less. A tail of 15 isolates (10.3%) had MICs of 2.5 or 5 mg/mL.

MBCs ranged from 0.156 to 10 mg/mL, with MBC50 0.625 mg/mL, MBC90 5 mg/mL and a geometric mean of 0.801 mg/mL. The most frequent MBC was 0.625 mg/mL (92 isolates, 63.4%). MBC was one dilution above MIC in 132 isolates (91.0%) and two dilutions above in 13 (9.0%), so the MBC/MIC ratio was 2 or 4 and never above 4.

Table 3. Distribution of *C. verum* MIC and MBC against 145 MDR *P. aeruginosa* isolates

Concentration (mg/mL)	MIC, n (%)	MIC cumulative %	MBC, n (%)	MBC cumulative %
0.078	6 (4.1)	4.1	0	0.0
0.156	9 (6.2)	10.3	5 (3.4)	3.4
0.312	104 (71.7)	82.1	10 (6.9)	10.3
0.625	10 (6.9)	89.0	92 (63.4)	73.8
1.25	1 (0.7)	89.7	22 (15.2)	89.0
2.5	12 (8.3)	97.9	1 (0.7)	89.7
5	3 (2.1)	100.0	12 (8.3)	97.9
10	0	100.0	3 (2.1)	100.0

Activity in relation to resistance profile and specimen

MIC was the same in resistant and susceptible isolates for every antibiotic (median 0.312 mg/mL in both groups), and no comparison was significant (smallest $P = 0.097$, for ceftazidime–avibactam; Table 2). This includes the 20 colistin-resistant isolates ($P = 0.14$) and the isolates resistant to imipenem ($P = 0.56$) or meropenem ($P = 0.61$). MIC did not correlate with the number of antibiotics resisted (Spearman $\rho = -0.10$, $P = 0.23$) or the number of resistant categories ($\rho = -0.05$, $P = 0.57$). The 15 isolates with MIC of 2.5 mg/mL or more resisted a median of 8 antibiotics, the same as the other isolates ($P = 0.80$).

MIC did not differ across specimen groups (Kruskal–Wallis $P = 0.96$; Table 4). MIC90 was 2.5 mg/mL for respiratory, urine and blood isolates and 0.312 mg/mL for pus and wound isolates, but the high-MIC isolates were spread over all four of these groups.

Table 4. *C. verum* MIC by specimen group

Specimen group	n	Median MIC (mg/mL)	MIC90 (mg/mL)	Isolates with MIC ≥2.5 mg/mL, n (%)
Pus, wound or other swab	57	0.312	0.312	3 (5.3)
Respiratory	33	0.312	2.5	4 (12.1)
Urine	31	0.312	2.5	5 (16.1)
Blood	13	0.312	2.5	3 (23.1)
Ear swab	5	0.312	0.625	0
Other	6	0.312	0.625	0
All isolates	145	0.312	2.5	15 (10.3)

DISCUSSION

In this series of 145 clinical MDR *P. aeruginosa* isolates, *C. verum* inhibited every isolate by broth microdilution, with a narrow, single-mode MIC distribution centred on 0.312 mg/mL, and killed them at one to two dilutions higher. Three findings stand out. Activity was bactericidal by the MBC/MIC criterion in all isolates [24]. It was independent of resistance to each of 13 antibiotics, including carbapenems and colistin. And it did not vary by specimen type.

Independence from antibiotic resistance

The lack of any association between *C. verum* MIC and antibiotic resistance is the most useful result. Resistance in *P. aeruginosa* rests on efflux, reduced porin permeability, AmpC and acquired β-lactamases, aminoglycoside-modifying enzymes and target-site mutations [2–4]. The antibacterial action of cinnamon is attributed mainly to cinnamaldehyde, which disrupts the cell membrane, inhibits cell division and enzymes, and interferes with quorum sensing [12–14]. These targets differ from those of the antibiotics tested, so cross-resistance is not expected, and our data show none. Two cautions

apply. The comparison was based on categorical S/R results, not antibiotic MICs. The dilution scale is also coarse: a one-step difference is the smallest effect the test can show. Even so, no trend emerged, and the colistin-resistant and carbapenem-resistant subsets had the same median MIC as susceptible isolates.

Comparison with earlier work

Our results agree in direction with reports that cinnamon bark oil and cinnamaldehyde inhibit MDR clinical *P. aeruginosa* and can act synergistically with antibiotics [15,16]. Direct numerical comparison is not possible. Published MICs for cinnamon come from essential oils, solvent extracts and pure cinnamaldehyde, are expressed in different units (% v/v, µg/mL or mg/mL), and often derive from small collections of isolates or agar diffusion [9,11,19]. A strength of the present study is a uniform, quantitative method on a large clinical collection with defined resistance phenotypes.

How strong is the activity?

A median MIC of 0.312 mg/mL equals 312 µg/mL. For crude plant extracts, MICs below 100 µg/mL are generally regarded as promising, 100–625 µg/mL as moderate and above 625 µg/mL as weak [25,26]. By this scheme, activity against most of our isolates (119 of 145, 82.1%, at ≤ 0.312 mg/mL) is moderate, six isolates (4.1%) fall in the promising range at 0.078 mg/mL, and 16 (11.0%) with MICs of 1.25–5 mg/mL are weak. These concentrations are far above those of conventional antibiotics, and we make no claim of therapeutic equivalence. The activity of a crude preparation also reflects its composition, which we did not measure.

The 10% tail of isolates needing 2.5–5 mg/mL was not explained by resistance phenotype or specimen. Possible reasons include strain differences in efflux of lipophilic compounds, outer membrane composition or other factors, but our data cannot test this and the explanation is a hypothesis for later study.

Translational considerations

In vitro inhibition at milligram-per-millilitre concentrations does not show that such levels can be reached safely in tissue. Cinnamaldehyde is volatile, binds to proteins and is cytotoxic at higher doses, and *C. verum* has less coumarin than *C. cassia* but is not free of safety questions [17]. The more realistic near-term roles are topical or local use (for example wound, ear or catheter-related infection, where our isolates were frequent), disinfection, and adjunctive use with antibiotics. Each requires its own evidence.

Strengths and limitations

Strengths are the large number of clinical isolates, a standardised reference method, MBC testing in addition to MIC, and linkage to a full resistance profile. Limitations are as follows. This was a single-centre study using one preparation, and the chemical composition (for example cinnamaldehyde content by GC-MS or HPLC) was not determined. Only planktonic cells were tested, and biofilm activity, time–kill kinetics, synergy with antibiotics and cytotoxicity were not studied. Antibiotic results were categorical, and resistance genes were not characterised. Two-fold dilution gives discrete, coarse MIC values. Future work should characterise the active constituents, test checkerboard synergy with colistin, carbapenems and newer β-lactam combinations, assess biofilm and in vivo models, and define the safety margin.

CONCLUSION

Cinnamomum verum inhibited and killed all 145 clinical MDR *P. aeruginosa* isolates by broth microdilution, with MIC50 0.312 mg/mL and MBC50 0.625 mg/mL, and its activity did not depend on resistance to carbapenems, colistin or any other antibiotic tested. The concentrations are moderate for a crude preparation, so these results justify chemical standardisation, synergy, biofilm and toxicity studies, and not yet clinical use.

Declarations

Ethics approval: The study received clearance from the Institutional Ethics Committee of Index Medical College Hospital and Research Center, affiliated with Malwanchal University, under approval number MU/Research/EC/Ph.D./2023/300.

Conflicts of interest: There is no any conflict of interest associated with this study.

Consent to participate: There is consent to participate.

Consent for publication: There is consent for the publication of this paper.

Authors & contributions: Author equally contributed the work.

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