ANTIBACTERIAL ACTIVITY OF *ANNONA MURICATA* LEAF BIOFRACTIONS AGAINST *PSEUDOMONAS AERUGINOSA* ISOLATED FROM WOUND INFECTIONS

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ABSTRACT
Wound infections caused by drug-resistant Gram-negative bacteria constitute a major global health concern, with *Pseudomonas aeruginosa* recognized as a leading etiological agent due to its intrinsic and acquired resistance mechanisms. The burden of antimicrobial resistance (AMR) associated with this pathogen exceeds 300,000 deaths annually, highlighting the urgent need for alternative therapeutic approaches. This study investigated the antibacterial activity of solvent-partitioned biofractions of *Annona muricata* (soursop) leaves against clinical isolates of *P. aeruginosa* obtained from wound infections. Fresh leaves were pulverized, extracted with methanol, and subsequently partitioned sequentially using n-hexane, ethyl acetate, and methanol. Ten isolates were confirmed using standard biochemical tests, while antibiotic susceptibility was determined using the disc diffusion method following Clinical and Laboratory Standards Institute (CLSI) guidelines. The antibacterial activity of the biofractions was evaluated at concentrations ranging from 100–400 µg/mL, and minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined using the broth microdilution method. Ethyl acetate yielded the highest extract (49.64%), followed by methanol (46.52%) and n-hexane (3.84%). All isolates exhibited multidrug resistance, although ciprofloxacin and gentamicin showed comparatively higher effectiveness. Both ethyl acetate and methanol fractions demonstrated concentration dependent antibacterial activity, with the ethyl acetate fraction showing slightly superior efficacy. MIC values ranged from 300–400 µg/mL, while MBC values were predominantly 400 µg/mL, indicating bactericidal effects. These findings support the potential of *A. muricata* leaf biofractions as promising candidates for the development of plant-based antimicrobial agents against resistant wound pathogens.

Keywords: *Annona muricata*; antimicrobial resistance; biofraction; disc diffusion; minimum inhibitory concentration; phytotherapy; *Pseudomonas aeruginosa*; wound infection

INTRODUCTION
Wound infections remain a principal source of morbidity in both community and hospital settings, increasing the risk of wound dehiscence, delayed healing, systemic sepsis, and mortality (Chang *et al*., 2016). Traumatic injuries constitute the predominant aetiological context for wound acquisition in hospitalised patients, yet healthcare-associated wounds including surgical incisions and in-dwelling device entry sites impose an equally significant burden (Bandy *et al*., 2022). The incidence of post-operative wound infections is disproportionately elevated in low- and middle-income countries, where gaps in infection prevention infrastructure, antibiotic stewardship, and laboratory capacity compound clinical outcomes (Tahir *et al*., 2025).

Pseudomonas aeruginosa, a non-fermenting, Gram-negative, aerobic opportunistic pathogen, is consistently implicated among the foremost causative agents of chronic wound infections, burn wound sepsis, and surgical site infections worldwide (Elfadadny *et al*., 2024). The organism demonstrates remarkable environmental resilience, persisting in diverse ecological niches including soil, water, hospital surfaces, plumbing systems, and the hands of healthcare workers, thereby facilitating nosocomial transmission (Pang *et al*., 2019). Its pathogenicity is sustained by a formidable arsenal of virulence determinants including exotoxins, proteases, siderophores, type III secretion system effectors, and the capacity for robust biofilm formation, each contributing to tissue destruction and immune evasion (Laborda *et al*., 2021).

The antimicrobial resistance profile of *P. aeruginosa* is among the most complex documented in clinical microbiology. Intrinsic mechanisms encompass reduced outer membrane permeability mediated by restricted porin expression, chromosomally encoded *AmpC* cephalosporinase inducibility, and constitutive expression of the MexAB-OprM efflux system. Acquired resistance is superimposed through plasmid-mediated extended-spectrum

β-lactamases, metallo-β-lactamases, aminoglycoside-modifying enzymes, and mutations affecting fluoroquinolone targets (Hancock & Speert, 2022; Lorusso *et al*., 2022; Oliver *et al*., 2024). The cumulative effect renders many clinical isolates extensively drug-resistant (XDR) or pan drug-resistant (PDR), leaving clinicians with severely constrained therapeutic options. The World Health Organization (WHO, 2021) designates *P. aeruginosa* as a Priority 1 critical-priority pathogen for which novel antibacterial agents are urgently required, a designation underscored by its annual global attributable mortality exceeding 300,000 deaths.

Against this backdrop, ethnopharmacologically informed plant-derived therapies have attracted intensified scientific scrutiny as sources of novel antibacterial scaffolds. Medicinal plants offer structurally diverse bioactive secondary metabolites, frequently exhibiting multi-target mechanisms of action that may circumvent single target resistance pathways operating in conventional antibiotics (Abba & Oduola, 2023). *Annona muricata* L. (Annonaceae), colloquially known as soursop, graviola, or guanabana, is a small evergreen tropical tree indigenising tropical Central and South America and West Africa, now widely distributed across tropical and subtropical regions globally (Coria-Téllez *et al*., 2018). The leaves of *A. muricata* are pharmacologically distinguished by a diverse phytochemical composition encompassing annonaceous acetogenins (AGEs), alkaloids, flavonoids, phenolic acids, and terpenoids, with documented antimicrobial, antioxidant, antiinflammatory, and antineoplastic activities (Mutakin *et al*., 2022; Moghadamtousi *et al*., 2015)

Ethnobotanical records from Nigeria, Ghana, and other West African nations describe the use of *A. muricata* leaf preparations, including teas, decoctions, and topical poultices, for the management of wounds, fevers, malaria, and respiratory infections (Kottila & Hena, 2024; Kumari *et al*., 2025; Nwonuma *et al*., 2023). In Southeast Asia, leaf poultices are applied directly to abscesses and wounds, leveraging anti-inflammatory and analgesic properties corroborated by pharmacological studies (Abdul Wahab *et al*., 2018; Haque *et al*., 2018). These cross-cultural ethnomedicinal traditions are reinforced by accumulating preclinical data demonstrating the inhibitory activity of *A. muricata* extracts against diverse bacterial species (Wilkie *et al*., 2023; Kazaure *et al*., 2024; Isaac *et al*., 2025). However, a critical gap persists in the literature regarding the antibacterial activity of systematically partitioned biofractions of *A. muricata* leaves against *P. aeruginosa* specifically isolated from wound infections. Solvent-solvent partitioning is a validated fractionation technique that enriches fractions with defined polarity ranges of phytochemical classes, enabling more precise attribution of antibacterial activity to specific metabolite groups and optimising extraction efficiency (Aguilar-Hernández *et al*., 2022; Nolasco-González *et al*., 2022). The present study, therefore, aimed to evaluate the antibacterial activity of n-hexane, ethyl acetate, and methanol biofractions of *A. muricata* leaves against *P. aeruginosa* clinical isolates, determine MIC and MBC values, and contextualise findings within the broader pharmacological and resistance literature.

MATERIALS AND METHODS

Study Area and Design

This study was conducted in the Department of Biology, Adeyemi Federal University of Education, Ondo, Ondo State, Nigeria. A cross-sectional, laboratory-experimental design was adopted, encompassing plant collection and fractionation, bacterial isolation and identification, antibiotic susceptibility testing, and quantitative antibacterial evaluation.

Plant Collection and Authentication

Fresh leaves of *Annona muricata* were collected from Igba, Ondo East Local Government Area, Ondo State, Nigeria. Taxonomic identification and authentication were conducted in the Herbarium Unit of the University of Medical Sciences (UNIMED), Ondo, and a voucher specimen was deposited under authentication number UNIMED/PBTH/0235.

Plant Preparation and Crude Extraction

Collected leaves were washed under running clean water, air-dried at ambient temperature for 27 days, and pulverised using an electric blender. Three hundred grams (300 g) of the powdered material were subjected to cold maceration in methanol for 72 hours with intermittent agitation. The extract was filtered through muslin cloth and the filtrate concentrated on a water bath at 50 °C. The crude extract was stored at 4 °C pending further processing, following the modified method of Ashagrie *et al*. (2023).

Solvent-Solvent Partitioning

Sequential fractionation was performed using the modified method of Kristiningrum *et al*. (2020). The crude methanolic extract was dissolved in 50 ml of distilled water and partitioned successively with 200 ml each of n-hexane, ethyl acetate, and methanol in a separating funnel, proceeding from the least to most polar solvent. Each fraction was collected, concentrated, weighed, and yield percentages calculated. Fractions were sterilised by passage through a 0.45 µm Millipore membrane filter after dissolving in 5% dimethyl sulphoxide (DMSO). Sterility was confirmed by inoculating 2 ml of each fraction into 10 ml Mueller-Hinton broth and incubating at 37 °C for 24 hours; absence of turbidity indicated sterility (Ojo *et al*., 2017). The hexane fraction was excluded from antibacterial assays owing to its critically low yield.

Identification of Test Organisms

Ten clinical isolates were obtained from the Department of Pharmaceutical Microbiology, University of Ibadan. Isolates were sub-cultured on cetrimide agar at 37 °C for 24 hours. Confirmation as *Pseudomonas aeruginosa* was based on colonial morphology, production of pyocyanin pigment with fruity odour, Gram staining, and a panel of biochemical tests including oxidase, catalase, motility, citrate utilisation, indole, urease, Voges-Proskauer, and methyl red tests (Cheesbrough, 2016).

Antibiotic Susceptibility Testing

Susceptibility testing was performed by the Kirby-Bauer disc diffusion method in accordance with CLSI (2023) breakpoints. A bacterial inoculum equivalent to 0.5 McFarland turbidity standard (approximately 1.5×10^6 CFU/ml) was prepared in sterile physiological saline. Eight commercial antibiotic discs were used: ciprofloxacin (5 µg), gentamicin (30 µg), ceftriaxone (30 µg), vancomycin (30 µg), amoxicillin (30 µg), oxacillin (5 µg), tetracycline (30 µg), and erythromycin (15 µg). Inoculated Mueller-Hinton agar plates were incubated at 37 °C for 24 hours and zones of inhibition measured and interpreted as sensitive (S), intermediate (I), or resistant (R). Antibacterial activity by disc diffusion working concentrations of 100, 200, 300, and 400 µg/ml of ethyl acetate and methanol fractions were prepared. Six-millimetre sterile Whatman filter paper discs were impregnated with the respective concentrations and placed on Mueller-Hinton agar seeded with 100 µl of 1×10^6 CFU/ml test organism. Plates were incubated at 37 °C for 24 hours. Zone of inhibition diameters were measured in independent triplicates.

Minimum Inhibitory Concentration (MIC)

MIC was determined by broth microdilution in 96-well microtitre plates following Appiah *et al*. (2017). Each well received 100 µL Mueller-Hinton broth, 50 µl biofraction (serial concentrations: 100–400 µg/ml), and 50 µL inoculum (1×10^6 CFU/ml). Following 24-hour incubation at 37 °C, 20 µl of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was added to each well. Pink coloration indicated bacterial growth; yellow or clear coloration indicated inhibition. The MIC was recorded as the lowest concentration producing no visible growth. All assays were performed in independent triplicates.

Minimum Bactericidal Concentration (MBC)

Following MIC determination, 50 µl aliquots from each well showing no visible growth were sub-cultured onto freshly prepared Mueller-Hinton agar and incubated at 37 °C for 24 hours. The MBC was defined as the lowest biofraction concentration yielding no colony growth, indicating bactericidal activity (Appiah *et al*., 2017). Assays were conducted in independent triplicates.

RESULTS

Extraction Yield of Annona muricata Biofractions

The solvent-solvent partitioning of the crude methanolic extract of *A. muricata* leaves yielded fractions whose masses and percentage yields are presented in Table 1. Ethyl acetate produced the highest yield (5.178 g; 49.64%), followed by methanol (4.853 g; 46.52%), while n-hexane yielded the smallest fraction (0.401 g; 3.84%).

Table 1: Weight and percentage yield of *Annona muricata* leaf biofractions

Solvent Used	Yield (g)	Yield (%)
n-Hexane	0.401	3.84

Ethyl Acetate	5.178	49.64
Methanol	4.853	46.52

Note: n-hexane fraction was excluded from bioassays due to insufficient yield

Biochemical Characterisation of Test Isolates

All ten clinical isolates were confirmed as *Pseudomonas aeruginosa* based on their consistent biochemical profiles (Table 2). Key features included Gram-negative morphology, positive oxidase and catalase reactions, positive motility, positive citrate utilisation, production of green pyocyanin pigment on cetrimide agar with characteristic fruity odour, and negative results for indole, urease, Voges-Proskauer, and methyl red tests.

Table 2: Biochemical characterisation profile of clinical *Pseudomonas aeruginosa* isolates

S/N	Biochemical Test	Result	Inference
1	Gram Staining	Negative	*P. aeruginosa*
2	Oxidase Test	Positive	*P. aeruginosa*
3	Catalase Test	Positive	*P. aeruginosa*
4	Motility Test	Positive	*P. aeruginosa*
5	Citrate Utilisation	Positive	*P. aeruginosa*
6	Indole Test	Negative	*P. aeruginosa*
7	Urease Test	Negative	*P. aeruginosa*
8	Voges-Proskauer Test	Negative	*P. aeruginosa*
9	Methyl Red Test	Negative	*P. aeruginosa*

Source: Authors' laboratory data (2024)

Antibiotic Susceptibility Profile

Antibiotic susceptibility testing against eight clinically relevant antibiotics revealed that ciprofloxacin exhibited the highest activity against the ten isolates (inhibition zones: 13–32 mm), followed by gentamicin (17–28 mm). Vancomycin produced variable results, while amoxicillin showed no inhibition against eight isolates. Tetracycline, oxacillin, and erythromycin demonstrated predominantly resistant or intermediate profiles. The complete susceptibility data are presented in Table 3.

Table 3: Antibiotic susceptibility profile of *P. aeruginosa* clinical isolates (zones of inhibition in mm)

S/N	Isolate	Gent (30µg)	Amox (30µg)	Tet (30µg)	Oxa (5µg)	Vanc (30µg)	Cro (30µg)	Cipro (5µg)	Eryth (15µg)
1	PA1	17(S)	NS	7(R)	7(R)	18(I)	20(S)	21(S)	9(R)
2	PA2	20(S)	NS	NS	11(R)	22(S)	16(I)	27(S)	25(S)
3	PA3	25(S)	NS	15(I)	NS	22(S)	21(S)	30(S)	20(S)
4	PA4	17(I)	NS	7(R)	7(R)	6(R)	26(S)	27(S)	7(R)
5	PA5	20(S)	NS	NS	11(R)	22(S)	16(I)	21(S)	23(S)
6	PA6	28(S)	NS	11(R)	5(R)	25(S)	15(I)	32(S)	17(I)
7	PA7	26(S)	15(I)	11(R)	NS	18(I)	23(S)	31(S)	NS
8	PA8	18(I)	25(S)	12(R)	11(R)	19(I)	17(I)	13(R)	21(S)
9	PA9	20(S)	NS	NS	11(R)	22(S)	16(I)	21(S)	23(S)
10	PA10	25(S)	25(I)	9(R)	15(R)	17(I)	20(S)	26(S)	25(S)

Key: I = Intermediate; R = Resistant; S = Susceptible; NS = No zone of inhibition. Gent = Gentamicin; Amox = Amoxicillin; Tet = Tetracycline; Oxa = Oxacillin; Vanc = Vancomycin; Cro = Ceftriaxone; Cipro = Ciprofloxacin; Eryth = Erythromycin

Antibacterial Activity of Biofractions by Disc Diffusion

Both the ethyl acetate and methanol fractions of *A. muricata* exhibited concentration-dependent inhibitory activity against all ten *P. aeruginosa* isolates (Tables 4a and 4b). Inhibition zones ranged from 3–10 mm for the ethyl acetate fraction and 4–10 mm for the methanol fraction across concentrations of 100–400 µg/ml. The ethyl acetate fraction demonstrated marginally superior activity at corresponding concentrations compared to the methanol fraction, particularly at 300 and 400 µg/ml.

Table 4a: Antibacterial activity of *A. muricata* ethyl acetate fraction against *P. aeruginosa* (disc diffusion; zones in mm)

S/N	Isolate	100 µg/ml	200 µg/ml	300 µg/ml	400 µg/ml
1	PA1	3	6	9	10
2	PA2	5	7	8	10
3	PA3	5	6	7	9
4	PA4	4	5	8	9
5	PA5	5	7	9	9
6	PA6	6	5	8	9
7	PA7	5	7	9	10
8	PA8	4	6	7	8
9	PA9	5	5	7	9
10	PA10	6	9	10	10

Source: Authors' laboratory data (2024)

Table 4b: Antibacterial activity of *A. muricata* methanol fraction against *P. aeruginosa* (disc diffusion; zones in mm)

S/N	Isolate	100 µg/ml	200 µg/ml	300 µg/ml	400 µg/ml
1	PA1	4	5	8	9
2	PA2	5	7	9	9
3	PA3	7	6	8	9
4	PA4	6	7	8	10
5	PA5	5	7	9	9
6	PA6	4	6	8	9
7	PA7	5	6	9	10
8	PA8	4	7	8	9
9	PA9	5	6	8	9
10	PA10	6	5	8	9

Source: Authors' laboratory data (2024)

Minimum Inhibitory and Bactericidal Concentrations

The MIC values for both ethyl acetate and methanol fractions against all ten isolates ranged between 300 and 400 µg/ml (Table 4c). MBC values, where determinable, were predominantly observed at 400 µg/ml for both fractions. Several isolates (PA1, PA4, PA5, PA6, PA9) did not yield a definitive MBC at the highest tested concentration, suggesting bacteriostatic rather than bactericidal activity at those concentrations for those particular isolates.

Table 4c: MIC and MBC values (µg/mL) of *A. muricata* biofractions against *P. aeruginosa* isolates

Isolate	EA MIC (µg/ml)	EA MBC (µg/ml)	MeOH MIC (µg/ml)	MeOH MBC (µg/ml)
PA1	300	–	400	–
PA2	300	–	400	400
PA3	300	400	400	400
PA4	400	–	400	–
PA5	400	–	400	–
PA6	400	400	400	–
PA7	300	400	300	400
PA8	300	400	300	400
PA9	400	NSI	400	–
PA10	300	400	300	400

Key: EA = Ethyl Acetate fraction; MeOH = Methanol fraction; NSI = No significant inhibition at tested concentrations; – = MBC not reached at 400 µg/ml.

DISCUSSION

Extraction Yield and Solvent Polarity

The differential extraction yields observed across solvents in this study reflect a well-established polarity dependent response in phytochemical extraction. The markedly superior yield of the ethyl acetate fraction

(49.64%) over n-hexane (3.84%) is consistent with the known chemical composition of *A. muricata* leaves, which are characterised by a predominance of semi-polar to polar secondary metabolites including acetogenins, flavonoid aglycones, terpenoids, and phenolic acids (Mutakin *et al*., 2022; Moghadamtousi *et al*., 2015). Ethyl acetate, as an intermediate-polarity solvent, is optimally suited for extracting this class of phytochemicals, whereas n-hexane preferentially solubilises lipophilic constituents such as waxes, chlorophylls, and fatty acids that are present in substantially smaller quantities in leaf material (Nawaz *et al*., 2022). The methanol fraction yield (46.52%) was marginally lower than ethyl acetate, likely reflecting the co-extraction of a higher proportion of polar non-antimicrobial compounds including sugars and amino acids, which dilute the concentration of pharmacologically active constituents per unit weight. These findings align with Sari *et al*. (2025) and Mishra *et al*. (2025), who independently documented polarity-dependent optimisation of *A. muricata* phytochemical recovery. The very low n-hexane yield corroborates earlier reports by Nawaz *et al*. (2022) establishing a consistent polarity-response pattern across diverse medicinal plant species.

Biochemical Identification of Pseudomonas aeruginosa

The biochemical panel utilised in this study produced a constellation of results entirely concordant with the established diagnostic profile of *P. aeruginosa*. The combination of Gram-negative morphology, positive oxidase reaction, positive motility, and citrate utilisation with negative results for indole, urease, Voges-Proskauer, and methyl red tests represents the classical phenotypic fingerprint of this species as described in contemporary reference manuals (Cheesbrough, 2016). The oxidase positivity warrants particular emphasis: the production of cytochrome c oxidase distinguishes *P. aeruginosa* unambiguously from members of the Enterobacteriaceae, which are characteristically oxidase-negative, providing an important rapid diagnostic criterion (Al-Daghistani *et al*., 2025). Production of pyocyanin on cetrimide selective agar further supports the identification, as pyocyanin is synthesised by approximately 90–95% of *P. aeruginosa* clinical isolates and serves as a species-specific marker. These biochemical findings are consistent with Ezeador *et al*. (2020), Al-Daghistani *et al*. (2025), and Wilkie *et al*. (2023), confirming that standard biochemical characterisation remains a reliable, accessible tool for identification in resource-limited settings.

Antibiotic Susceptibility and Resistance Mechanisms

The antibiotic susceptibility patterns observed in this study reflect the characteristic multidrug-resistant phenotype of *P. aeruginosa* described extensively in contemporary literature. Ciprofloxacin demonstrated the broadest and strongest activity (zones of inhibition: 13–32 mm), a finding consistent with the fluoroquinolone mechanism of inhibiting DNA gyrase (GyrA/GyrB) and topoisomerase IV to prevent DNA replication and transcription (Wu et *al*., 2024). Gentamicin exhibited the second-greatest activity by binding the 30S ribosomal subunit to cause misreading of mRNA codons, disrupting translational fidelity. The pattern is congruent with the report of Elfadadny *et al*. (2024), who documented fluoroquinolones and aminoglycosides as retaining greater clinical utility against *P. aeruginosa* compared to β-lactams, particularly aminopenicillins. The near-universal resistance to amoxicillin observed in this study is mechanistically explicable. *P. aeruginosa* intrinsically produces chromosomally encoded AmpC β-lactamase, which efficiently hydrolyses aminopenicillins. This is compounded by reduced outer membrane permeability owing to the constitutive downregulation of the OprD porin, and the operation of the MexAB-OprM efflux system, which actively expels β-lactam antibiotics, macrolides, and fluoroquinolones (Lorusso *et al*., 2022). The convergence of these mechanisms in clinical isolates from wound infections is particularly concerning from a therapeutic standpoint. Oliver *et al*. (2024), in a comprehensive European survey, documented that XDR profiles were increasingly common among wound associated *P. aeruginosa*, limiting treatment to last-resort agents such as colistin or novel β lactam/β-lactamase inhibitor combinations. The pronounced resistance observed in the current isolates reinforces the local and global urgency of identifying alternative antibacterial strategies.

Antibacterial Activity of Biofractions

Both the ethyl acetate and methanol fractions of *A. muricata* demonstrated inhibitory activity against all ten clinical *P. aeruginosa* isolates, with inhibition zones increasing proportionally with concentration. The dose-dependent concentration-response relationship confirms fundamental pharmacological coherence and is consistent across multiple prior studies on *A. muricata* antibacterial activity (Wilkie *et al*., 2023; Nolasco-González *et al*., 2022; Kazaure *et al*., 2024). The marginally higher activity of the ethyl acetate fraction compared

to the methanol fraction is consistent with the enrichment of semi-polar antimicrobial compounds principally acetogenins, flavonoid aglycones, and terpenoids in the ethyl acetate partition. Bushirat *et al*. (2025) quantified flavonoids (8.5–12.1%) and alkaloids (10.2–15.6%) differentially distributed across ethyl acetate, ethanol, and aqueous fractions of *A. muricata*, demonstrating that fraction polarity directly governs the relative abundance of pharmacologically active compound classes. The precise molecular mechanisms by which *A. muricata* phytochemicals inhibit *P. aeruginosa* are incompletely characterised, but available evidence points to several complementary actions. Annonaceous acetogenins have been shown to inhibit mitochondrial NADH-ubiquinone oxidoreductase (Complex I) and disrupt bacterial membrane integrity, leading to loss of transmembrane potential and cell lysis (Coria-Téllez *et al*., 2018; Kottila & Hena, 2024). Flavonoids such as quercetin and rutin interfere with cell wall synthesis, DNA gyrase activity, and membrane fluidity (Mutakin *et al*., 2022). Alkaloids may interact with nucleic acids and inhibit topoisomerase activity. The multi-target nature of these phytochemical classes is particularly relevant in the context of *P. aeruginosa* resistance, as the probability of simultaneous resistance mutations at multiple pharmacological targets is substantially lower than for single-target agents. Dey *et al*. (2025) corroborated these findings through GC-MS profiling of *A. muricata* bark fractions, identifying terpenoids and fatty acid derivatives as key contributors to antibacterial activity against multidrug-resistant pathogens.

MIC and MBC Interpretation

The MIC values obtained in this study (300–400 µg/ml) are higher than those reported for purified acetogenin preparations from *A. muricata*, where Aguilar-Hernández *et al*. (2022) documented MIC values as low as 0.009–12.5 µg/ml for isolated compounds, compared to 12.5–4000 µg/ml for crude extracts. This underscores a fundamental pharmacological principle: the presence of phytochemically complex matrices in crude fractions dilutes the concentration of individual active principles, reducing apparent potency per unit mass. Fractionation as employed in the present study represents an intermediate step, enriching but not purifying individual active compounds. The logical pharmacological extension is bioassay-guided isolation of the principal antibacterial constituents to determine which specific molecular entities confer the observed activity and at what concentrations. The predominantly bactericidal profile suggested by MBC values at 400 µg/ml in multiple isolates particularly the ethyl acetate fraction, is pharmacodynamically relevant. A bactericidal MBC:MIC ratio of ≤4 is conventionally used to define bactericidal rather than bacteriostatic activity. Where MBC values were determinable in this study, the MBC:MIC ratio for several isolates satisfies this criterion. Bactericidal activity is particularly desirable for wound infection management, where high bacterial burden and impaired host immunity create conditions where bacteriostatic agents may be insufficient to achieve clinical resolution. The heterogeneous response across isolates (some failing to yield a determinable MBC at 400 µg/ml) likely reflects inter-isolate variation in efflux pump expression and membrane permeability, mirroring the phenotypic diversity documented by Oliver *et al*. (2024) and Wu *et al*. (2024).

CONCLUSION

This study demonstrates that solvent-partitioned biofractions of *Annona muricata* leaves, particularly the ethyl acetate fraction, exhibit significant concentration-dependent antibacterial activity against multidrug-resistant clinical isolates of *Pseudomonas aeruginosa* obtained from wound infections. The ethyl acetate fraction showed the highest extraction yield and marginally superior antibacterial activity, which may be attributed to its selective enrichment of semi-polar bioactive constituents. Minimum inhibitory concentration (MIC) values of 300–400 µg/ml and predominantly bactericidal minimum bactericidal concentration (MBC) values of 400 µg/ml indicate pharmacodynamically relevant activity, supporting the ethnomedicinal use of the plant in wound management. These findings provide a scientifically grounded basis for further investigation of *A. muricata* as a potential source of novel plant-derived antimicrobial agents.

RECOMMENDATIONS

Based on the findings of this study, the following recommendations are made for advancing the scientific and clinical translation of *Annona muricata* as an antimicrobial resource:

i. Bioassay-Guided Phytochemical Fractionation: The ethyl acetate and methanol fractions yielding the most promising antibacterial activity should be subjected to successive column chromatography and preparative high performance liquid chromatography (HPLC) to isolate, purify, and structurally characterise individual bioactive

constituents, particularly acetogenins, flavonoid aglycones, and terpenoids. Structural elucidation by nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry is strongly recommended.

ii. Mechanism-of-Action Studies: Molecular and cellular investigations should be conducted to elucidate the precise mechanisms by which *A. muricata* phytochemicals inhibit *P. aeruginosa*. Recommended approaches include bacterial membrane integrity assays, efflux pump inhibition studies, biofilm disruption experiments, and genomic or proteomic analyses to identify molecular targets. This will clarify whether observed activity is mediated through membrane disruption, inhibition of cell wall synthesis, interference with metabolic enzymes, or multi-target effects.

iii. *In vivo* Pharmacological Evaluation: Compelling in vitro antibacterial activity reported here should be validated in appropriate animal infection models (e.g., murine wound infection or *Galleria mellonella* larvae models) to assess in vivo efficacy, pharmacokinetics, and acute toxicity. Such studies are indispensable to bridge the gap between in vitro findings and potential clinical application, and to determine bioavailability and tissue distribution of active fractions.

iv. Combination Studies with Conventional Antibiotics: Given the intrinsic multidrug resistance of *P. aeruginosa*, studies evaluating synergistic or additive interactions between *A. muricata* fractions and conventional antibiotics (particularly ciprofloxacin and gentamicin) are warranted. Checkerboard MIC assays and time-kill kinetics should be employed to determine whether phytochemical fractions can potentiate the activity of existing antibiotics or overcome resistance mechanisms including efflux pump activity.

v. Expanded Clinical Isolate Diversity: Future studies should include a larger number and greater geographic diversity of *P. aeruginosa* clinical isolates, encompassing XDR and PDR strains characterised by specific resistance genotypes (e.g., metallo-β-lactamase producers, hypermutable strains), to better define the breadth and limits of the antibacterial activity of *A. muricata* fractions.

vi. Safety and Toxicological Profile: Systematic preclinical toxicological evaluation, including cytotoxicity assays on mammalian cell lines, haemolytic activity assays, and sub-acute toxicity studies in rodent models, must precede any clinical application. These studies are critical to establish the therapeutic index and safety window of identified active fractions, particularly for topical wound application where direct tissue contact must be characterised.

vii. Formulation Research: In parallel with pharmacological validation, pharmaceutical formulation research is recommended to develop stable, biocompatible delivery systems for *A. muricata* active fractions suitable for wound application, such as topical gels, dressings, or nanoparticle-based delivery systems that can enhance local bioavailability and maintain therapeutic concentrations at the wound site.

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