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Original Research Article

Aqueous Leaf Extract of *Cotoneaster nummularia* Fisch. & C.A.Mey. Partially Inhibits the Growth of *Escherichia coli* and *Pseudomonas aeruginosa* Isolated from Hospital Waste: A Broth Microdilution Study

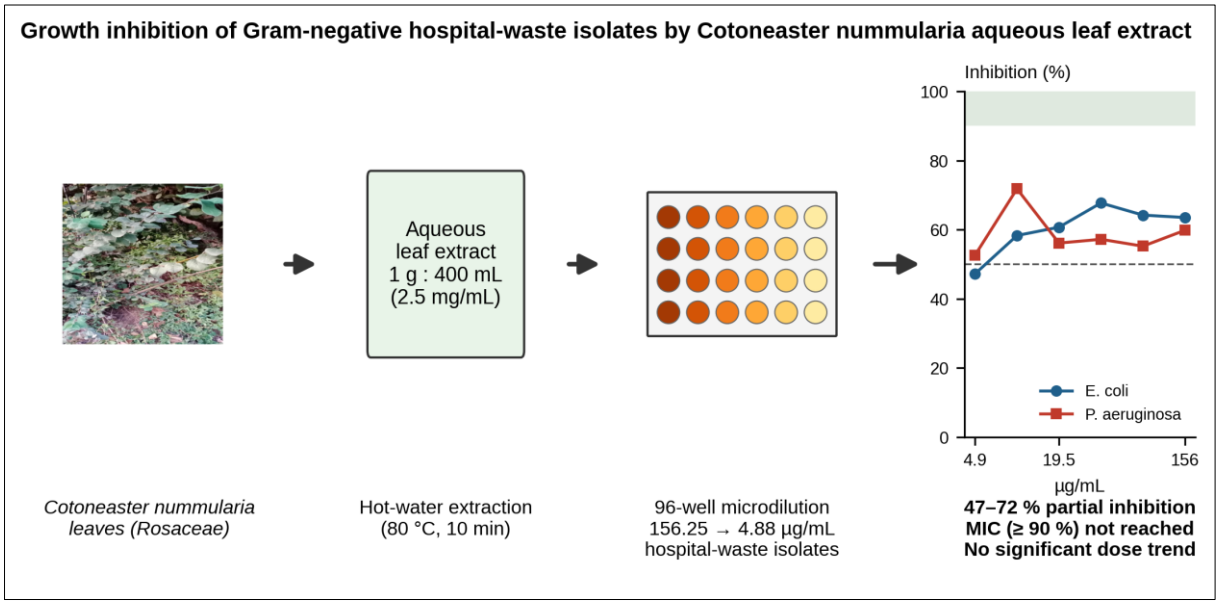
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Abstract



Graphical abstract. Aqueous *Cotoneaster nummularia* leaf extract was tested by broth microdilution against *E. coli* and *P. aeruginosa* from hospital waste; partial inhibition (47–72 %) was observed without reaching the MIC criterion.

Escherichia coli and *Pseudomonas aeruginosa* are leading causes of drug-resistant infection and are listed among the World Health Organization priority pathogens. Species of *Cotoneaster* (Rosaceae) are used in traditional Asian medicine and are rich in polyphenols, but evidence for activity of *Cotoneaster nummularia* against Gram-negative bacteria of clinical or environmental origin is scarce. Leaves of *C. nummularia* were collected in Dir Lower, Khyber Pakhtunkhwa, Pakistan, and extracted with water (1g: 400 mL; 24 h maceration followed by 10 min at 80 °C). *E. coli* and *P. aeruginosa* were isolated from dry waste of Saidu Teaching Hospital, Swat. Growth inhibition was measured by 96-well broth microdilution

over a two-fold series of 4.88–156.25 µg/mL (leaf-equivalent) by optical density at 600 nm after 24 h at 37 °C. The minimum inhibitory concentration (MIC) was defined a priori as the lowest concentration producing ≥ 90 % inhibition. Concentration–response association was assessed by Spearman correlation and between-isolate differences by the Wilcoxon signed-rank test. The extract partially inhibited both isolates at every concentration tested (*E. coli* 47.2–67.7 %; *P. aeruginosa* 52.6–71.8 %). No concentration reached 90 % inhibition; the MIC therefore exceeded 156.25 µg/mL for both organisms. Inhibition of ≥ 50 % was observed from 9.77 µg/mL for *E. coli* and at all concentrations for *P. aeruginosa*. Inhibition was not significantly associated with concentration (*E. coli* $\rho = 0.77$, $p = 0.07$; *P. aeruginosa* $\rho = 0.26$, $p = 0.62$), and mean inhibition did not differ between isolates (difference −1.5 percentage points; $p = 0.84$). Aqueous *C. nummularia* leaf extract exerts a partial, plateau-type growth-inhibitory effect on Gram-negative hospital-waste isolates but does not achieve MIC-level inhibition within the tested range.

Keywords: *Cotoneaster nummularia*; Rosaceae; aqueous extract; broth microdilution; minimum inhibitory concentration; *Escherichia coli*; *Pseudomonas aeruginosa*; hospital waste; antimicrobial resistance; polyphenols.

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1. INTRODUCTION

Antimicrobial resistance (AMR) is now one of the leading causes of death worldwide. A systematic analysis of 204 countries estimated that, in 2019, bacterial AMR was directly responsible for 1.27 million deaths and associated with 4.95 million deaths, and that six pathogens, led by *Escherichia coli* and including *Pseudomonas aeruginosa*, accounted for most of this burden (Murray *et al.,* 2022; Ather *et al.,* 2026). Forecasts extending to 2050 indicate that, without effective intervention, attributable deaths will continue to rise, with the steepest increases expected in South Asia (Naghavi *et al.,* 2024). This growing burden has not been matched by an adequate pipeline of new antibacterial agents (Tacconelli *et al.,* 2018).

Gram-negative bacteria dominate the priority lists that guide antibacterial research and development. The first World Health Organization (WHO) priority pathogen list placed carbapenem-resistant *P. aeruginosa* and carbapenem-resistant or third-generation cephalosporin-resistant Enterobacteriaceae, including *E. coli*, in its critical category (Tacconelli *et al.,* 2018), and the 2024 update retained resistant Enterobacterales in the critical tier and resistant *P. aeruginosa* among the high-priority pathogens (World Health Organization, 2024). These organisms are difficult targets because their outer membrane restricts the entry of many hydrophobic and large hydrophilic molecules (Nikaido, 2003). *P. aeruginosa* combines an outer membrane of unusually low permeability with multiple chromosomally encoded efflux systems, inducible β-lactamases and a capacity for adaptive and mutational resistance, so that almost every antibiotic class is eventually compromised (Breidenstein *et al.,* 2011; Pang *et al.,* 2019; Faazal *et al.,* 2023; Chaudhary *et al.,* 2025). Any candidate antibacterial, synthetic or natural, must therefore be judged against the permeability barrier that Gram-negative bacteria present.

Hospitals are hotspots for the selection and dissemination of resistant bacteria. Hospital effluents and wastes contain extended-spectrum β-lactamase-producing *E. coli* and resistant *P. aeruginosa* at densities that depend on local clinical prevalence, and the antimicrobial residues they carry exert continuous selective pressure downstream (Hocquet *et al.,* 2016). In low- and middle-income settings, where segregation, treatment and disposal of hospital waste are frequently incomplete, such waste can act as a bridge between clinical and community reservoirs. Isolates recovered from hospital waste therefore represent a relevant, if under-studied, test population for new antibacterial candidates.

Natural products remain the most productive historical source of antibacterial scaffolds. Of the small-molecule drugs approved between 1981 and 2019, a large proportion were natural products or were derived from or inspired by them (Newman & Cragg, 2020), and advances in analytical chemistry, genome mining and screening technologies have renewed interest in plant-derived leads (Atanasov *et al.,* 2021; Qasim *et al.,* 2026). Plants produce phenolic acids, flavonoids, tannins, terpenoids and alkaloids with documented antimicrobial activity (Cowan, 1999). Polyphenols, in particular, can damage the cytoplasmic membrane, chelate metal ions, inhibit enzymes and interfere with virulence factors (Daglia, 2012); flavonoids act on nucleic acid synthesis, membrane function and energy metabolism (Cushnie & Lamb, 2005), and tannins complex with proteins and deprive bacteria of iron (Scalbert, 1991; Ullah *et al.,* 2026). However, many of these compounds are considerably more active against Gram-positive than against Gram-negative bacteria (Taguri *et al.,* 2004), which is consistent with the outer-membrane barrier described above.

The value of the plant antimicrobial literature is limited by methodological heterogeneity. Reviews have repeatedly noted that crude extracts are often tested at concentrations that have no pharmacological meaning, without standardised inocula, positive controls or replicate measurements, and with endpoints that are not comparable across studies (Cos *et al.,* 2006; Ríos & Recio, 2005; Qasim *et al.,* 2026; Haidri *et al.,* 2026). Broth microdilution is the reference method for determining the minimum inhibitory concentration (MIC) of antimicrobial substances (Clinical and Laboratory Standards Institute [CLSI], 2018; International Organization for Standardization [ISO], 2019; Wiegand *et al.,* 2008), and colorimetric indicators such as tetrazolium salts or resazurin have been adapted to overcome the turbidity and colour of plant extracts (Eloff, 1998; Sarker *et al.,* 2007). Diffusion assays, by

contrast, are poorly suited to extracts whose constituents diffuse unequally through agar (Klančnik *et al.,* 2010). Commonly used activity thresholds classify extracts with MIC values below 100 µg/mL as significant, 100–625 µg/mL as moderate and above 625 µg/mL as weak (Kuete, 2010; Kuete & Efferth, 2010), while extracts active only above 1 mg/mL are generally considered of little interest (Ríos & Recio, 2005).

The genus *Cotoneaster* Medik. (Rosaceae) comprises several hundred species distributed across temperate Eurasia, a number of which are used in traditional Asian medicine for haemorrhoids, diabetes, cardiovascular disorders, fever and wound care (Kicel, 2020). More than 90 constituents have been identified in *Cotoneaster* leaves, fruits, flowers and twigs, dominated by flavan-3-ols and procyanidins, flavonol glycosides, caffeoylquinic and other phenolic acids, triterpenes and cyanogenic glycosides (Kicel, 2020; Kicel *et al.,* 2016). Extracts of several species show antioxidant, anti-inflammatory and enzyme-inhibitory activities (Kicel *et al.,* 2018, 2019; Uysal *et al.,* 2016), and anti-acne activity against skin bacteria has been reported for two cultivated species (Krzemińska *et al.,* 2022).

Cotoneaster nummularia Fisch. & C.A.Mey. is a shrub of the mountainous regions of South-West and Central Asia, including northern Pakistan. Zengin et al. (2014) characterised water, methanol and ethyl acetate extracts of its leafy twigs, identified ferulic acid, chlorogenic acid, (−)-epicatechin and (+)-catechin as the major phenolics and reported MIC values of 0.625 mg/mL for the water extract against *Staphylococcus aureus*, including methicillin-resistant strains. More recently, a polar leaf extract of the species was used to synthesise silver nanoparticles with antimicrobial activity (Assad *et al.,* 2023; Qasim *et al.,* 2025). To our knowledge, the activity of aqueous *C. nummularia* extract, the preparation closest to traditional decoctions, against Gram-negative bacteria recovered from a hospital environment has not been quantified with a microdilution method and pre-defined endpoints.

The aim of the present study was therefore to measure the growth-inhibitory activity of an aqueous leaf extract of *C. nummularia* against *E. coli* and *P. aeruginosa* isolated from hospital dry waste in Swat, Pakistan. We specified in advance that (i) the MIC would be defined as the lowest concentration producing ≥ 90 % growth inhibition, (ii) a concentration-dependent effect would be accepted only if inhibition was significantly and positively associated with concentration, and (iii) a difference in susceptibility between the two organisms would be accepted only if it was statistically supported. Stating these criteria explicitly is intended to avoid the over-interpretation of partial inhibition that is common in this field (Cos *et al.,* 2006; Qasim *et al.,* 2026). The work re-analyses and extends preliminary data first reported in a master's thesis (Khan & Ullah, 2021).

2. MATERIALS AND METHODS

2.1. Chemicals, media and equipment

Nutrient agar was used for the isolation, purification and maintenance of bacterial cultures, and liquid growth medium (broth) was used in the microdilution assay. Assays were performed in sterile flat-bottom 96-well microtiter plates, and optical density was measured with a microplate reader. All reagents were of analytical grade, and distilled water was used throughout.

2.2. Plant material

Fresh leaves of *Cotoneaster nummularia* Fisch. & C.A.Mey. (Rosaceae) were collected from Dir Lower, Khyber Pakhtunkhwa, Pakistan, from wild-growing shrubs (Figure 1A). The plant material was authenticated by a taxonomist at the Center for Plant Sciences and Biodiversity, University of Swat, Pakistan. Only leaves were harvested, and whole plants were not uprooted.

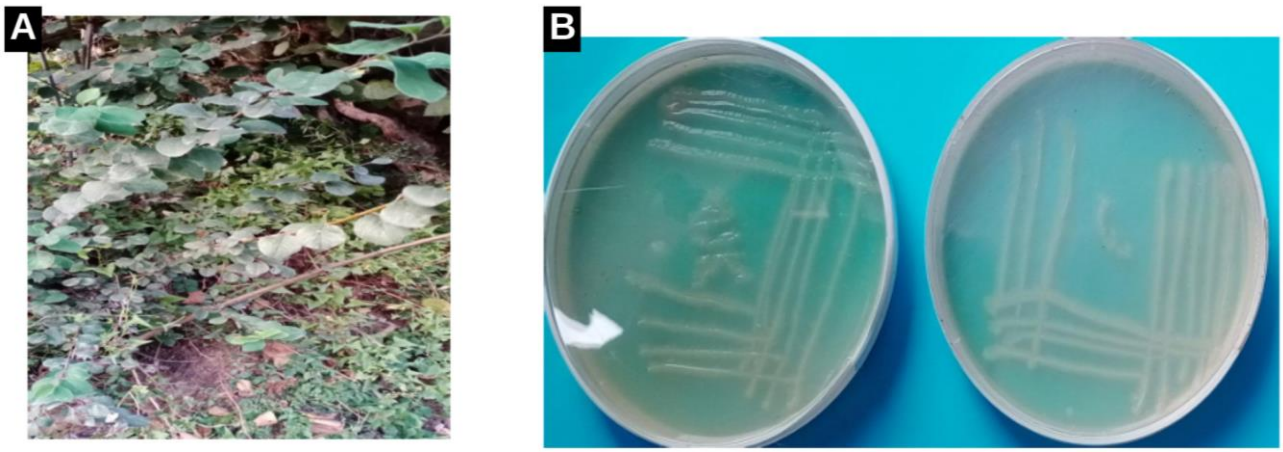


Figure 1: (A) *Cotoneaster nummularia* in its natural habitat at the collection site in Dir Lower, Khyber Pakhtunkhwa, Pakistan, from which leaves were harvested. (B) Fresh cultures of the *Escherichia coli* hospital-waste isolate on nutrient agar after 24 h at 37 °C.

2.3. Preparation of the aqueous extract

Leaves were washed with tap water followed by distilled water, shade-dried at ambient temperature for 14 days and ground to a fine powder. One gram of powder was macerated in 400 mL of distilled water for 24 h at room temperature and then heated at 80 °C for 10 min to mimic a traditional hot-water preparation. The mixture was filtered through Whatman filter paper and stored in sterile containers until use. The filtrate was not passed through a membrane filter before testing.

Because the dry mass of extracted solids was not determined, all concentrations are expressed as leaf-equivalents, i.e., micrograms of dried leaf powder extracted per milliliter (µg/mL). The primary filtrate therefore corresponded to 2.5 mg/mL (2,500 µg/mL). Because the solute content of an aqueous leaf extract is only a fraction of the leaf mass, the concentration of extracted material in the assay wells was lower than these nominal values.

2.4. Bacterial isolates

Clinical-environmental isolates of *E. coli* and *P. aeruginosa* were obtained from dry waste collected at Saidu Teaching Hospital, Swat, Khyber Pakhtunkhwa, Pakistan. The isolates were cultured on nutrient agar and incubated at 37 °C for 24 h to obtain fresh, pure cultures (Figure 1B), which were used for all assays. One isolate of each species was tested. The confirmatory identification tests applied to the isolates are not reported here, and this is acknowledged as a limitation (Section 4.8).

2.5. Inoculum preparation

Colonies from fresh 24 h cultures on nutrient agar were used to prepare a standardized bacterial suspension, which was used to inoculate each test well. The reference broth microdilution method specifies a final inoculum of approximately 5×10^5 CFU/mL (CLSI, 2018; Wiegand *et al.,* 2008); the cell density of the inoculum was not verified by viable count in the present study.

2.6. Broth microdilution assay

Growth inhibition was determined in 96-well plates following the general principles of the broth microdilution method (CLSI, 2018; ISO, 2019). The extract was serially diluted two-fold in broth to give final in-well concentrations of 156.25, 78.13, 39.06, 19.53, 9.77 and 4.88 µg/mL. Each test well received the standardized inoculum. Control wells without extract served as the reference for 0 % inhibition. Extract-only wells (color blanks), a reference antibiotic and CLSI quality-control strains were not included in this experiment. Plates were incubated at 37 °C for 24 h, and optical density was read at 600 nm (OD_{600}). Each concentration was tested as a single series, yielding one inhibition value per concentration and isolate.

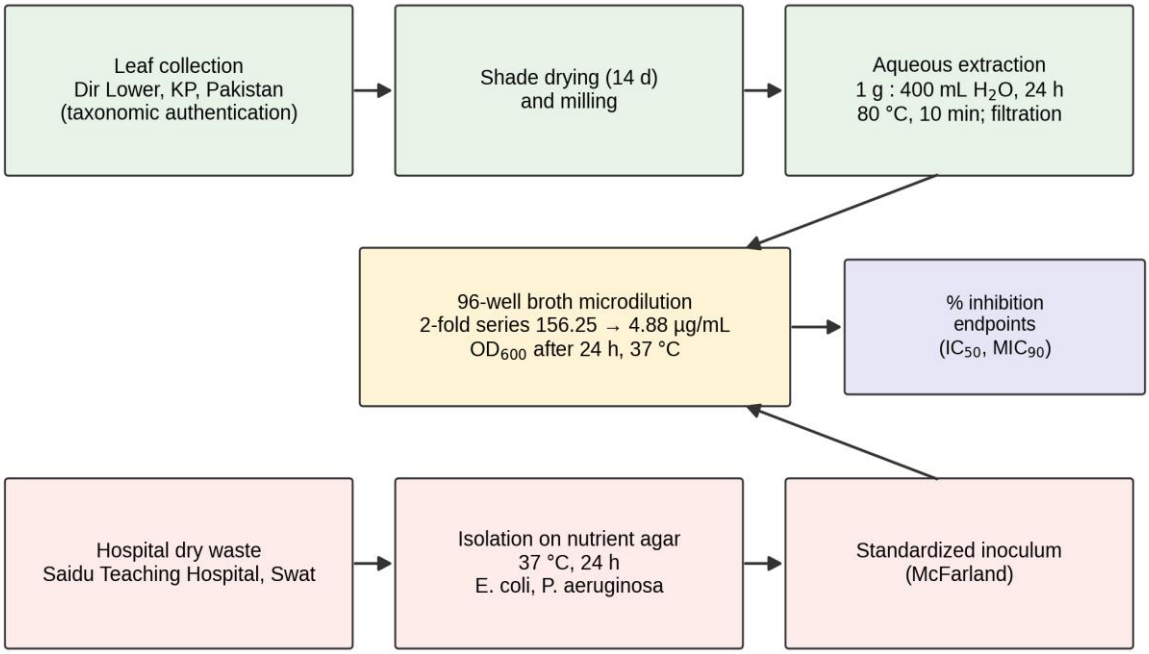


Figure 2: Experimental workflow. Leaves were dried, milled and extracted with water; bacterial isolates were recovered from hospital dry waste and standardized; growth inhibition was measured by broth microdilution over a two-fold concentration series and read at 600 nm. Endpoints were defined before analysis (MIC, ≥ 90 % inhibition)

Table 1: Extraction and assay conditions used in this study, compared with reference recommendations for broth microdilution

Parameter	Condition used in this study	Reference recommendation
Plant part and origin	Leaves, Dir Lower, Khyber Pakhtunkhwa, Pakistan	Voucher specimen deposited; accepted name verified
Solvent and ratio	Distilled water; 1 g: 400 mL (2.5 mg/mL leaf-equivalent)	Report extraction yield (% w/w)
Extraction	24 h maceration at room temperature; 80 °C for 10 min	—
Clarification	Whatman filter paper	Sterile 0.22 µm filtration or sterility check
Concentration range	Two-fold series, 156.25–4.88 µg/mL	Range should bracket the MIC
Medium	Broth growth medium	CAMHB (CLSI, 2018)
Final inoculum	Standardised suspension (density not verified)	≈ 5×10^5 CFU/mL (CLSI, 2018; ISO, 2019)
Incubation	37 °C, 24 h	35 ± 2 °C, 16–20 h (CLSI, 2018)
Read-out	OD_{600}	OD with colour correction, or resazurin / tetrazolium indicator
Controls	Untreated control (0 % inhibition)	Growth, sterility, colour, antibiotic and QC-strain controls
Replication	Single series	≥ 3 technical replicates × ≥ 2 independent experiments

2.7. Calculation of growth inhibition and definition of endpoints

Percentage growth inhibition was calculated for each concentration as:

$$\text{Inhibition (\%)} = [1 - (OD_{test} / OD_{control})] \times 100$$

where OD_{test} is the reading of the extract-treated well and $OD_{control}$ that of the control well without extract. Because colour blanks were not included, the readings were not corrected for the absorbance of the extract itself; the implications of this are considered in Section 4.3.

Three endpoints were defined before analysis. The MIC was defined as the lowest concentration producing ≥ 90 % inhibition, a threshold commonly adopted for spectrophotometric readings when visual endpoints are unavailable (Balouiri *et al.,* 2016; Wiegand *et al.,* 2008). If no tested concentration met this criterion, the MIC was reported as greater than the highest concentration tested. The lowest concentration producing ≥ 50 % inhibition was recorded as a descriptor of partial activity; because the concentration series did not bracket the 50 % level for *P. aeruginosa*, a formal IC_{50} could not be interpolated. The maximum observed inhibition and the concentration at which it occurred were also recorded.

2.8. Statistical analysis

Because each concentration was represented by a single value, parametric curve fitting was not attempted. The association between concentration and inhibition was tested for each isolate with Spearman's rank correlation coefficient (ρ; $n = 6$). Differences in inhibition between the two isolates across matched concentrations were tested with the two-sided Wilcoxon signed-rank test, with a paired *t*-test as a sensitivity analysis. Statistical significance was set at $\alpha = 0.05$. Analyses were performed in Python 3 using the SciPy library.

3. RESULTS

3.1. Extract and test concentrations

The aqueous extraction yielded a primary filtrate corresponding to 2.5 mg of dried leaf per mL, from which the six test concentrations (4.88–156.25 µg/mL) were obtained by two-fold dilution (Table 1; Figure 2). The highest concentration tested thus corresponded to a 16-fold dilution of the primary filtrate.

3.2. Growth inhibition of *E. coli*

The extract inhibited the growth of the *E. coli* isolate at all six concentrations, with inhibition ranging from 47.2 % at 4.88 µg/mL to 67.7 % at 39.06 µg/mL (Table 2; Figure 3). Inhibition rose from 47.2 % to 60.7 % between 4.88 and 19.53 µg/mL and then remained between 63.5 % and 67.7 % at 39.06–156.25 µg/mL, a plateau-type profile in which a further four-fold increase in concentration produced no additional inhibition. The lowest concentration producing at least 50 % inhibition was 9.77 µg/mL. The rank correlation between concentration and inhibition was positive but not statistically significant ($\rho = 0.77$, $p = 0.07$; Table 3).

Table 2: Growth inhibition of hospital-waste isolates of *Escherichia coli* and *Pseudomonas aeruginosa* by the aqueous leaf extract of *Cotoneaster nummularia*

Concentration (µg/mL)	*E. coli* inhibition (%)	*P. aeruginosa* inhibition (%)	Difference, *P. aeruginosa* − *E. coli* (pp)
156.25	63.5	59.8	−3.7
78.13	64.2	55.2	−9.0
39.06	**67.7**[a]	57.2	−10.5
19.53	60.7	56.1	−4.6
9.77	58.3	**71.8**[a]	+13.5
4.88	47.2	52.6	+5.4
Untreated control	0.0	0.0	—
Mean ± SD (6 concentrations)	60.3 ± 7.2	58.8 ± 6.8	−1.5 ± 9.2

Values are single determinations per concentration from a single experimental series; SD in the last row describes variation across concentrations, not experimental error. [a] Maximum observed inhibition. None of the values reaches the ≥ 90 % MIC criterion. pp, percentage points.

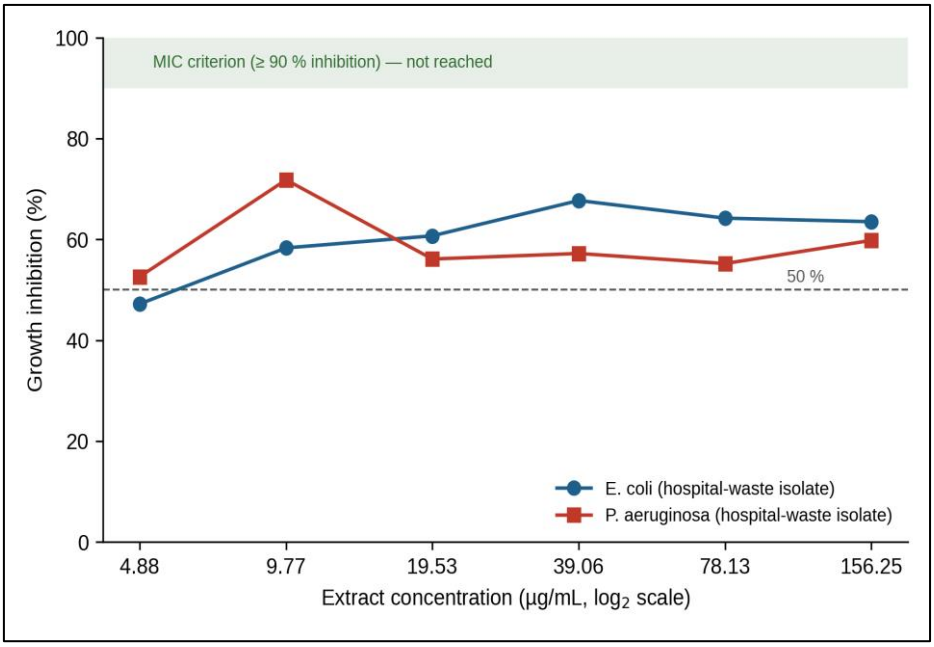


Figure 3: Concentration–response profiles of *Escherichia coli* and *Pseudomonas aeruginosa* hospital-waste isolates exposed to aqueous *Cotoneaster nummularia* leaf extract. The shaded band marks the ≥ 90 % inhibition criterion for the MIC, which was not reached; the dashed line marks 50 % inhibition. Points are single determinations; concentrations are leaf-equivalents on a log₂ scale

3.3. Growth inhibition of *P. aeruginosa*

Inhibition of the *P. aeruginosa* isolate ranged from 52.6 % at 4.88 µg/mL to 71.8 % at 9.77 µg/mL (Table 2; Figure 3). The profile was non-monotonic: after the maximum at 9.77 µg/mL, inhibition fell to 55.2–59.8 % across 19.53–156.25 µg/mL, so that the 16-fold higher concentration produced 12 percentage points less inhibition than 9.77 µg/mL. All six concentrations produced more than 50 % inhibition; the 50 % level was therefore not bracketed, and the lowest concentration producing ≥ 50 % inhibition is reported as ≤ 4.88 µg/mL. There was no significant association between concentration and inhibition ($\rho = 0.26$, $p = 0.62$).

3.4. Comparison between isolates

Inhibition was higher for *P. aeruginosa* than for *E. coli* at the two lowest concentrations (+5.4 and +13.5 percentage points) but lower at the four highest concentrations (−3.7 to −10.5 percentage points) (Table 2; Figure 4). Averaged over the six concentrations, inhibition was 60.3 % for *E. coli* and 58.8 % for *P. aeruginosa* (mean paired difference −1.5 percentage points). Neither the Wilcoxon signed-rank test ($p = 0.84$) nor the paired *t*-test ($t(5) = -0.39$, $p = 0.71$) indicated a difference between the isolates (Table 3). The data therefore do not support the conclusion that one organism was more susceptible than the other.

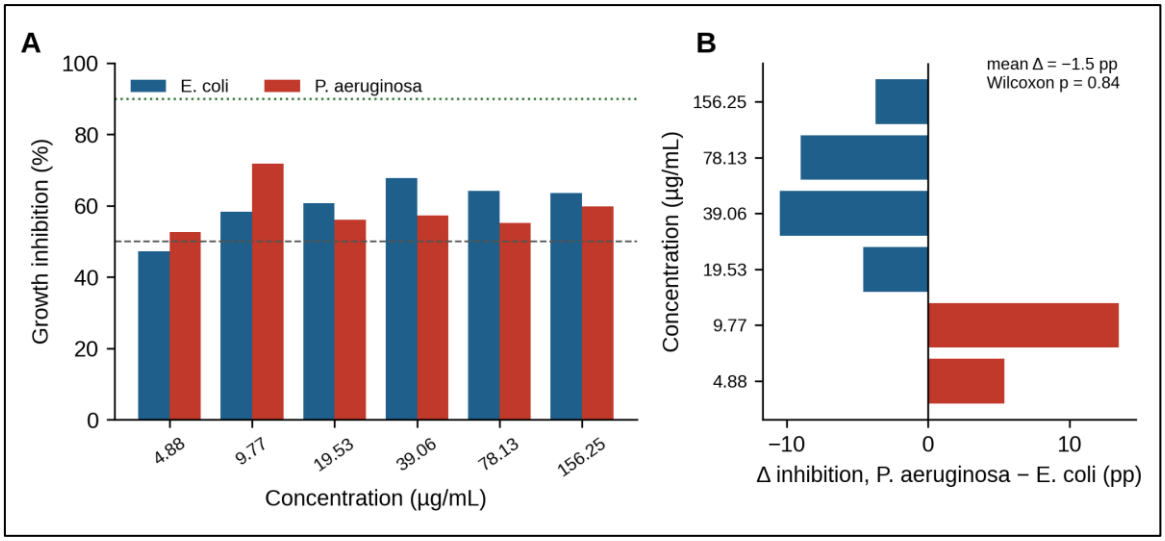


Figure 4: Comparison between isolates. (A) Growth inhibition at each concentration; dotted line, 90 %; dashed line, 50 %. (B) Paired differences in inhibition (*P. aeruginosa* − *E. coli*) at each concentration; positive values indicate greater inhibition of *P. aeruginosa*. The mean difference was −1.5 percentage points (Wilcoxon signed-rank test, $p = 0.84$)

Table 3: Summary endpoints and statistical tests

Endpoint	*E. coli*	*P. aeruginosa*
Range of inhibition (%)	47.2–67.7	52.6–71.8
Maximum inhibition (%) [concentration]	67.7 [39.06 µg/mL]	71.8 [9.77 µg/mL]
Lowest concentration with ≥ 50 % inhibition	9.77 µg/mL	≤ 4.88 µg/mL (not bracketed)
MIC (≥ 90 % inhibition)	> 156.25 µg/mL	> 156.25 µg/mL
Spearman ρ, concentration vs. inhibition	0.77 ($p = 0.07$)	0.26 ($p = 0.62$)
Between-isolate comparison	Wilcoxon signed-rank $p = 0.84$; paired $t(5) = -0.39$, $p = 0.71$	

3.5. Minimum inhibitory concentration

No tested concentration achieved the pre-specified MIC criterion of ≥ 90 % inhibition for either organism; the highest value recorded for any condition was 71.8 %. The MIC was therefore greater than 156.25 µg/mL (leaf-equivalent) for both *E. coli* and *P. aeruginosa* (Table 3). The lowest concentration tested, 4.88 µg/mL, should not be interpreted as an MIC: inhibition there was only 47.2 % (*E. coli*) and 52.6 % (*P. aeruginosa*), meaning that roughly half of the control growth persisted.

4. DISCUSSION

4.1. Principal findings

This study quantified the growth-inhibitory activity of an aqueous leaf extract of *C. nummularia* against Gram-negative isolates recovered from hospital dry waste. The extract reduced bacterial growth by roughly one-half to two-thirds at every concentration between 4.88 and 156.25 µg/mL, but it did not approach complete inhibition, and the degree of inhibition was essentially independent of concentration over a 32-fold range. By the criteria set before analysis, the extract did not reach an MIC within the tested range, no concentration dependence was demonstrated, and no difference in susceptibility between *E. coli* and *P. aeruginosa* was found. The data are consistent with a modest, partial effect on bacterial growth; they are not consistent with potent antibacterial activity.

4.2. Partial inhibition is not an MIC

The MIC is, by definition, the lowest concentration of an agent that prevents visible growth of a standardized inoculum under defined conditions (CLSI, 2018; ISO, 2019; Wiegand *et al.,* 2008). When growth is read spectrophotometrically, a threshold of 80–90 % reduction in optical density relative to the growth control is usually taken as the equivalent of no visible growth (Balouiri *et al.,* 2016). A concentration at which roughly half of the control turbidity persists is therefore, by any standard, a concentration at which the organism grows. Reporting the lowest concentration tested as the MIC, merely because some inhibition was measurable there, conflates the MIC with a threshold of detectable effect and would, if accepted, make every extract appear potent simply by extending the dilution series downwards. Because our dilution series began at 156.25 µg/mL and never produced ≥ 90 % inhibition, the correct statement is that the MIC lies above 156.25 µg/mL. Whether it lies at 312.5 µg/mL, 2.5 mg/mL or beyond cannot be determined from these data, and the concentration range should be extended upwards in future work.

Placing this result on the scales commonly used for plant extracts is informative. Extracts with MIC values below 100 µg/mL are usually regarded as significantly active and those above 625 µg/mL as weak (Kuete, 2010; Kuete & Efferth, 2010), and activity at concentrations above 1 mg/mL is generally considered of little pharmacological relevance (Ríos & Recio, 2005). The present extract falls outside the 'significant' category by definition, and its classification as moderate or weak awaits testing at higher concentrations. Moreover, concentrations here are expressed as leaf-equivalents; the dry mass of solutes in an aqueous extract of leaves is typically a fraction of the leaf mass, so the concentration of extracted material in the wells was lower than the nominal values. Reporting the extraction yield would allow direct comparison with studies that express concentrations as dry extract.

4.3. Interpreting the flat and non-monotonic concentration–response

A genuine antibacterial agent tested over a two-fold series normally produces a sigmoidal concentration–response curve that rises from little or no effect to complete inhibition across a relatively narrow range. The profiles observed here differ in two ways: they are flat, with 47–72 % inhibition at every concentration, and in the case of *P. aeruginosa* they are non-monotonic, with maximal inhibition at the second-lowest concentration. Several explanations, not mutually exclusive, should be considered before these profiles are attributed to biology.

First, optical interference. Aqueous leaf extracts rich in polyphenols are colored and can form precipitates with broth proteins, particularly at higher concentrations. If the absorbance of the extract itself is not subtracted, or if precipitates form during incubation, OD_{600} readings can be biased in either direction, compressing the apparent concentration–response. This problem is well recognised in the plant antimicrobial literature and is the reason why redox indicators such as p-iodonitrotetrazolium violet or resazurin, which report metabolic activity rather than turbidity, were introduced for microplate testing of extracts (Eloff, 1998; Elshikh *et al.,* 2016; Sarker *et al.,* 2007; Qasim et al., 2026). Second, measurement error. With a single determination per concentration, a difference of 10–15 percentage points between adjacent concentrations cannot be distinguished from well-to-well variation, which in turbidimetric microplate assays is often of this order. The isolated maximum of 71.8 % for *P. aeruginosa* at 9.77 µg/mL is exactly the kind of value that replicate testing may fail to reproduce. Third, genuine biphasic behaviour. Low-dose stimulation or high-dose attenuation of biological responses, described under the general heading of hormesis (Calabrese & Baldwin, 2003), and concentration-dependent aggregation of polyphenols that reduces their availability are both documented phenomena; however, invoking them requires reproducible data, which are not yet available.

The direction of the colour artefact is worth stating explicitly, because it bears directly on the interpretation of the present data. In this experiment no extract-only blanks were read, so any absorbance contributed by the extract at 600 nm was counted as bacterial growth. Such absorbance increases with extract concentration, and it therefore depresses the calculated inhibition most strongly at the highest concentrations. An extract whose true antibacterial effect increased steadily with concentration could, under these conditions, produce exactly the flat or declining profile observed here, and the true inhibition at 78.13 and 156.25 µg/mL may have been underestimated. Conversely, precipitation of extract constituents in the well can scatter light and has the same effect, while partial sedimentation of such precipitates before reading could act in the opposite direction. The present data cannot distinguish between these possibilities, which is why the absence of a concentration–response relationship should be treated as a property of the measurement as much as of the extract. Repeating the assay with colour blanks at every concentration and with a metabolic indicator such as resazurin would resolve the question directly and at little cost.

A flat inhibition profile may also indicate a bacteriostatic effect that slows growth without preventing it, so that turbidity after 24 h is reduced but not abolished. Time–kill assays and determination of the minimum bactericidal concentration by sub-culture would distinguish slowed growth from killing, and should be included in any follow-up study.

4.4. Comparison with previous work on *Cotoneaster*

Our findings are broadly consistent with the limited existing evidence. The most complete previous study of *C. nummularia* reported an MIC of 0.625 mg/mL for the water extract against *S. aureus*, including methicillin-resistant strains, using broth microdilution (Zengin *et al.,* 2014). That value, obtained against Gram-positive bacteria that lack an outer membrane, is four times higher than the highest concentration tested here. It would therefore be surprising if the extract reached an MIC against Gram-negative bacteria at concentrations below 156.25 µg/mL, and the absence of an MIC in the present study is what the prior literature would predict. Other reports on the genus concern different species or preparations, such as nanoparticles synthesized with *C. nummularia* extract (Assad *et al.,* 2023) or anti-acne activity against skin bacteria (Krzemińska *et al.,* 2022), and the review by Kicel (2020) notes that antimicrobial data for *Cotoneaster* are heterogeneous in methodology. None of these studies provides evidence that aqueous *C. nummularia* extract is potently active against *E. coli* or *P. aeruginosa* (Table 4).

Table 4: Previously reported antibacterial activity of *Cotoneaster* preparations compared with the present study

Species / preparation	Method	Organisms	Main finding	Reference
C. nummularia, water, methanol and ethyl acetate extracts of leafy twigs	Broth microdilution	*S. aureus* (MSSA, MRSA), *Sarcina lutea* and other strains	Water extract MIC 0.625 mg/mL against *S. aureus* (MSSA, MRSA) and *S. lutea*	Zengin et al. (2014)
C. nummularia, polar leaf extract used to synthesise silver nanoparticles	Antimicrobial assays of nanoparticles	Three bacterial and two fungal strains	Nanoparticles, not the extract alone, showed antimicrobial activity	Assad et al. (2023)
Two cultivated *Cotoneaster* species	Anti-acne assays	Acne-associated bacteria	Anti-acne potential reported	Krzemińska et al. (2022)
Genus *Cotoneaster* (review)	—	Various	Antimicrobial activity among reported effects; data heterogeneous	Kicel (2020)
C. nummularia, aqueous leaf extract	Broth microdilution, OD_{600}	*E. coli*, *P. aeruginosa* (hospital waste)	47–72 % inhibition; MIC > 156.25 µg/mL (leaf-equivalent)	Present study

The major phenolics of *C. nummularia* extracts, namely chlorogenic acid, ferulic acid, (−)-epicatechin and (+)-catechin (Zengin *et al.,* 2014), offer a plausible chemical basis for partial inhibition. Chlorogenic acid increases outer- and plasma-membrane permeability and causes leakage of cytoplasmic contents (Lou *et al.,* 2011); ferulic and gallic acids alter membrane hydrophobicity, charge and integrity in both Gram-positive and Gram-negative bacteria (Borges *et al.,* 2013); and catechins and tannins bind membrane proteins and chelate iron (Daglia, 2012; Scalbert, 1991). These mechanisms, however, generally operate at concentrations of individual compounds that are high relative to those achievable in a dilute crude aqueous extract, and polyphenols are typically more active against Gram-positive than Gram-negative species (Taguri *et al.,* 2004). Because the extract used here was not chemically characterized, any attribution of activity to particular constituents remains speculative.

4.5. No evidence of differential susceptibility

It is tempting to interpret the higher inhibition of *P. aeruginosa* at 9.77 µg/mL as evidence of greater susceptibility and to explain it by differences in cell envelope structure. The data do not support this. *P. aeruginosa* was inhibited more than *E. coli* at two concentrations and less at four; the average difference was 1.5 percentage points in favour of *E. coli*, and formal tests found no difference. Furthermore, the structural argument points the other way: the outer membrane of *P. aeruginosa* is considerably less permeable than that of *E. coli*, and the organism possesses extensive efflux capacity (Breidenstein *et al.,* 2011; Nikaido, 2003; Pang *et al.,* 2019). If anything, *P. aeruginosa* would be expected to be less susceptible to plant polyphenols. A claim of differential susceptibility would require replicate data, several isolates of each species and an appropriate statistical comparison.

4.6. Relevance of hospital-waste isolates

Testing isolates from hospital waste is a strength of the study design, because such isolates are more likely than reference strains to carry acquired resistance determinants and reflect the local resistance ecology (Hocquet *et al.,* 2016). This strength can only be realized if the isolates are properly characterized. Species identification must be documented, and an antibiotic susceptibility profile interpreted with current CLSI breakpoints (CLSI, 2024) is needed before the isolates can be described as resistant or multidrug-resistant under the internationally agreed definitions (Magiorakos *et al.,* 2012). Testing a single isolate per species also limits generalization, since susceptibility to plant extracts can vary between strains; a panel of isolates, together with reference strains (*E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853), would allow both generalization and quality control.

4.7. Traditional use, safety and translational outlook

Hot-water preparations of *Cotoneaster* leaves and twigs are used in folk medicine across the range of the genus (Kicel, 2020), and the aqueous extract tested here was deliberately prepared to resemble such a decoction. From an ethnopharmacological standpoint, the relevant question is not whether a crude extract can be made to produce some inhibition in a microplate, but whether the concentrations reached by a traditional preparation could plausibly affect bacteria at the site of infection. The primary filtrate in this study corresponded to 2.5 mg of leaf per mL, and even this undiluted preparation would need to be at least 16-fold more active than the highest concentration tested to reach an MIC. For systemic infections caused by *E. coli* or *P. aeruginosa*, the concentrations of polyphenols achievable in plasma after oral intake are far lower still, because many flavan-3-ols and phenolic acids are poorly absorbed and extensively metabolized. Any realistic application would therefore be topical or local, for example in wound care, where the antioxidant and anti-

inflammatory properties reported for *C. nummularia* extracts (Zengin *et al.,* 2014) might also contribute.

Safety must be considered alongside efficacy. Cyanogenic glycosides have been reported in several *Cotoneaster* species, and the toxicological data for the genus are limited (Kicel, 2020). Before any preparation is proposed for human use, the extract should be chemically standardized, screened for cyanogenic constituents, and tested for cytotoxicity against mammalian cells so that a selectivity index can be calculated (Cos *et al.,* 2006). Positive findings from in vitro screening of crude extracts have a poor record of translation into clinical antibacterial, largely because the active constituents are rarely identified and are often present at concentrations too low to be useful (Cos *et al.,* 2006; Ríos & Recio, 2005). Bioassay-guided fractionation of *C. nummularia* extracts, followed by testing of pure constituents against characterized Gram-negative isolates, would be the most direct way to determine whether the partial inhibition observed here reflects a single potent compound diluted in the crude extract or the combined weak effects of common polyphenols. The possibility of synergy with conventional antibiotics, which does not require the extract to be potent on its own, is a further avenue that could be pursued with checkerboard assays.

4.8. Limitations

The study has important limitations that constrain its conclusions. (i) Each concentration was tested once, so experimental variability and reproducibility are unknown. (ii) The concentration range did not extend high enough to bracket the MIC. (iii) The read-out relied on OD_{600} without correction for extract absorbance. (iv) No reference antibiotic or quality-control strain was reported, so assay performance cannot be verified. (v) Only one isolate of each species was tested, the confirmatory identification tests are not reported, and no antibiotic susceptibility profile was determined, so the isolates cannot be classified as resistant or multidrug-resistant. (vi) The extract was not chemically characterized or standardized, and concentrations are expressed as leaf-equivalents. (vii) Bactericidal activity, cytotoxicity to mammalian cells and in vivo efficacy were not assessed.

4.9. Recommendations for Future Work

A study capable of establishing whether *C. nummularia* has meaningful activity against Gram-negative pathogens should (i) test at least three technical replicates in two or more independent experiments; (ii) extend the concentration range to at least 2.5 mg/mL and express concentrations as dry extract; (iii) use CAMHB, a standardized inoculum of 5×10^5 CFU/mL and CLSI quality-control strains; (iv) include a reference antibiotic, growth, sterility and extract-color controls, and confirm endpoints with resazurin or a tetrazolium indicator; (v) determine minimum bactericidal concentrations and time–kill kinetics; (vi) test a panel of characterized clinical and environmental isolates; (vii) characterize the extract by total phenolic content (Singleton & Rossi, 1965) and HPLC or LC–MS profiling; and (viii) assess cytotoxicity to establish a selectivity index (Cos *et al.,* 2006). A plate layout that satisfies points (i)–(iv) is provided in Supplementary Table S1. Combination testing with conventional antibiotics may also be worthwhile, since sub-inhibitory concentrations of plant polyphenols can potentiate antibiotic activity against Gram-negative bacteria.

5. CONCLUSIONS

The aqueous leaf extract of *Cotoneaster nummularia* reduced the growth of *Escherichia coli* and *Pseudomonas aeruginosa* isolated from hospital waste by 47–72 % over a concentration range of 4.88–156.25 µg/mL, but it did not achieve MIC-level (≥ 90 %) inhibition, showed no significant concentration dependence and did not discriminate between the two organisms. The MIC is therefore greater than 156.25 µg/mL for both isolates. These preliminary, single-series data indicate at most a modest growth-inhibitory effect and do not justify describing the extract as a potential alternative to antibiotics. Replicated, standardized testing with appropriate controls, antibiotic comparators, characterized isolates and chemically defined extracts is required to determine whether this traditional medicinal plant has antibacterial activity of practical relevance against Gram-negative pathogens.

Declarations

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethics Statement

The study did not involve human participants, human tissue or animals. Bacterial isolates were recovered from hospital dry waste from discarded hospital dry waste; no patient specimens and no patient-identifiable information were collected. All work with bacterial cultures was performed under Biosafety Level 2 conditions.

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Highlights

- An aqueous leaf extract of *Cotoneaster nummularia* was tested against *Escherichia coli* and *Pseudomonas aeruginosa* recovered from hospital dry waste.
- The extract produced partial growth inhibition (47.2–71.8 %) at all concentrations from 4.88 to 156.25 µg/mL.
- No concentration reached the ≥ 90 % inhibition criterion, so the MIC lies above 156.25 µg/mL for both isolates.
- Inhibition was not significantly related to concentration and did not differ between the two isolates.
- Replicated, CLSI-conformant testing with antibiotic comparators is required before any therapeutic claim.

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