Study of the Inhibitory Effect of Myrtle (*Myrtus communis* L.) Leaf Powder Extracts on *Escherichia coli* and *Pseudomonas aeruginosa*

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Abstract

With the emergence of antimicrobial resistance of Gram-negative pathogens worldwide, the need for plant-derived antibacterials has increased. Myrtle (Myrtus communis L., Myrtaceae) is a shrub native of the Mediterranean area, with leaves reported as antimicrobial due to their high content in phenolics and terpenes. In this study, the inhibitory potential of M. communis leaves extracts on two Gram-negative pathogens namely E. coli and P. aeruginosa which are of clinical and food importance was evaluated in vitro. Three extraction methods namely cold aqueous maceration, hot aqueous extraction and 70% ethanolic extraction were used to extract air-dried powdered leaves and the extraction yields were determined gravimetrically (13.4, 19.2 and 20.6% w/w, respectively). Qualitative phytochemical screening revealed the presence of tannins, phenolics, flavonoids and glycosides in all extracts with terpenoids and resins being enriched in ethanolic fraction. The agar well diffusion method was used to evaluate each extract at five concentrations (1.2, 2.5, 5, 12 and 24% w/v) against both organisms in three independent replicates (a 3 × 5 × 2 factorial arrangement; 90 extract determinations in total), and the data were analysed by factorial ANOVA and by non-parametric comparison with the reference standards. Sterile distilled water was used for the negative control and gentamicin (10 µg) and ciprofloxacin (5 µg) as the positive reference standards. Extracts at all concentrations showed no zone of inhibition against either organism (6.0 ± 0.0 mm including the well; 0 mm net inhibition), whereas the reference antibiotics produced clear and reproducible zones (17.3-28.3 mm; $P < 0.001$ versus all extract treatments) confirming that the assay was sufficiently sensitive to detect inhibition. Because only a diffusion format was used, minimum inhibitory concentrations were not determined and low-level activity detectable by dilution methods cannot be excluded. The absence of detectable activity is most plausibly attributable to the Gram-negative outer-membrane permeability barrier, the limited agar diffusion of mainly non-polar constituents of myrtle and also by the known limitations of agar diffusion assays, and is consistent with previous studies on myrtle that reported no diffusion-detectable activity against E. coli and only weak, dilution-detectable activity against Pseudomonas.

Keywords: *Myrtus communis; myrtle; antibacterial activity; Escherichia coli; Pseudomonas aeruginosa; agar well diffusion; phytochemical screening; Gram-negative bacteria; plant extracts*

1. Introduction

1.1 Background

Bacterial infectious diseases continue to be one of the biggest killers and sickness agents globally and antimicrobial resistance (AMR) is an increasing challenge for their management. AMR is now known to be one of the leading causes of mortality and morbidity due to drug resistant bacterial infections, with the estimated toll of millions of deaths caused by such infections each year [2]. Antibiotic-resistant Gram-negative bacteria are once

ISSN 2072-3857

again included in the World Health Organization's 2024 Bacterial Priority Pathogens List, as a list of pathogens for which novel treatments are urgently needed [3]. This has led to increased interest in the search of alternative and complementary antibacterial agents, especially those of natural origin that have been used by man for a long time [1, 4].

Medicinal plants have always been a valuable source of bioactive secondary metabolites and plant-derived antimicrobials are still being studied for their possible role in the discovery of new drugs as well as their use in the food industry as natural preservatives [1], [5], [32]. The antibacterial, antifungal and antioxidant properties are commonly attributed to the phenolic compounds, flavonoids, tannins and terpenoid constituents of essential oils [1], [31], [34]. This interest is based on the consumer demand for natural products, and also on the possibility of having structurally diverse phytochemicals that can interact with targets in bacteria other than those targeted by traditional antibiotics [27], [33].

Myrtle (Myrtus communis L.), a member of the Myrtaceae Family, is an evergreen shrub that is native to the Mediterranean basin and found in many parts of the Middle East, both as a wild plant and an ornamental [6,7]. The leaves, berries and volatile oils have been used as antiseptic, anti-inflammatory, astringent and antimicrobial agents in traditional medicine for a long time [7], [8]. The leaves contain high concentrations of phenolic compounds including acylphloroglucinols: myrtucommulone A, B and semi-myrtucommulone, flavonoids and tannins, while the essential oil is mainly composed of monoterpenes including 1,8-cineole, α-pinene, limonene and linalool [8] and [9]. Some of these components showed antibacterial properties in vitro and myrtle extracts have been suggested as potential natural antimicrobial agents [8, 10].

However, the antibacterial spectrum reported for M. communis is not consistent. A number of studies report high activity against Gram-positive bacteria (Staphylococcus aureus and Listeria monocytogenes) and against acid fast bacteria, but lack or low activity against Gram-negative bacteria [8, 10, 15]. This difference is indicative of a more general trend that Gram-negative bacteria are more resistant to crude plant extracts than Gram-positive bacteria, which has been well documented [21]–[22] and is believed to be due to the extra outer membrane of Gram-negative bacteria that prevents the penetration of many hydrophobic and amphipathic phytochemicals [35].

These are two organisms that we studied here that represent this challenge. Escherichia coli is a Gram-negative, facultative anaerobe bacteria family member of Enterobacteriaceae, which normally resides as a commensal in the intestinal tract, but can acquire virulence factors to cause several types of infection including UTI, diarrhoeal disease, neonatal meningitis and bloodstream infections [17]. P. aeruginosa is an omnipresent, obligate aerobic, Gram negative bacterium, which is an important opportunistic nosocomial pathogen in the clinical setting due to its intrinsic and acquired resistance to various antibiotic classes, production of pigments and virulence factors, and ability to persist in clinical environments [18, 19]. It has an intrinsic resistance due to low OM permeability, constitutive and inducible efflux pumps, and β-lactamase production, and is one of the most difficult pathogens to treat [19, 20].

1.2 Research gap

Although there has been considerable literature on M. communis, there are some gaps. Most positive reports are about the essential oils or organic-solvent (methanol, ethanol, ethyl-acetate) extractions, and comparative data on simple aqueous extractionthe type most likely to be used in resource-limited and household settingsare less abundant and less consistent [8], [10], [14]. Second, there are conflicting data regarding the effectiveness of these for the

Gram-negative organisms E. coli and P. aeruginosa, with some investigators showing measurable inhibition, and others showing none, under apparently similar conditions [10, 11]. Third, a significant amount of screening is carried out using the agar diffusion format which while convenient is not suitable for non-polar plant constituents and is known for false negative and non-reproducible results [24, 26]. Last but not least, negative results are under-reported, thus skewing the evidence base to apparent effectiveness [24]. A need therefore exists for well-controlled studies with definite results from the assessment of the performance of defined myrtle extracts against these pathogens, including adverse effects, validated assay controls and phytochemical characterization.

1.3 Aim and objectives

The aim of this study was to evaluate the in vitro inhibitory effect of *M. communis* leaf powder extracts on *E. coli* and *P. aeruginosa* using the agar well-diffusion method. The specific objectives were:

(i) to prepare and characterize three distinct leaf extractscold aqueous, hot aqueous and 70% ethanolicincluding determination of extraction yield and qualitative phytochemical composition;

(ii) to assess the antibacterial activity of each extract against E. coli and P. aeruginosa across five concentrations (1.2-24% w/v) with three independent replicates per treatment;

(iii) to validate the sensitivity of the assay using a sterile-distilled-water negative control and standard antibiotic reference discs (gentamicin and ciprofloxacin);

(iv) to analyze the data statistically and to compare the outcome directly with previously published myrtle studies; and

(v) to interpret the result in the context of Gram-negative cell-envelope physiology, extract chemistry and the limitations of diffusion-based screening.

1.4 Contributions

The principal contributions of this work are: (i) a transparent, fully replicated evaluation of three extracts from myrtle leaves against 2 priority Gram-negative pathogens, with a clear negative result together with validated positive and negative controls, helping to counter the bias in reporting towards positive results in plant-antimicrobial studies [24]; (ii) a documented negative dataset including extraction yields, phytochemical screening and complete, replicate-level inhibition data, to help counter the bias in reporting toward positive results in plant-antimicrobial studies [24]; (iii) an explicit, tabulated comparison of the present findings with previous studies of myrtle, clarifying where these agree and differ; and (iv) a machine-readable supplementary dataset, by which every measurement can be independently verified.

2. Materials and Methods

2.1 Study design and setting

The study was experimental, in vitro, carried out in the Microbiology and food-science laboratories of the Department of Food Sciences, College of Agriculture, University of Kufa, Najaf, Iraq. The experiment was laid out as a completely randomized design with a 3 × 5 × 2 factorial arrangement of three factorsextract type (three levels), concentration (five levels) and test organism (two levels)with three independent replicates per treatment combination, giving 90 extract determinations in addition to the negative-control and reference-antibiotic determinations (Section 2.8). The corresponding factorial statistical analysis is described in Section 2.9. A list of instruments and chemicals is given in Appendix A (Tables A1 and A2).

2.2 Plant collection, identification and preparation of leaf powder

ISSN 2072-3857

The leaves of M. communis were collected from a domestic garden in the city of Al-Diwaniyah, Iraq at vegetative stage, when they were fresh and healthy. Based on the morphological features of the plant, which are the usual characteristics of the species: evergreen habit, small opposite, lanceolate to ovate, glossy, aromatic leaves and white star-shaped flowers, the plant was authenticated as Myrtus communis L. (Myrtaceae). The leaves were separated from the stems, dust and extraneous material was removed and the leaves were washed with distilled water. These were then spread in a single layer, and dried in the shade at room temperature, for about ten days, away from direct sunlight to minimize loss of thermolabile and volatile constituents [30]. The dried leaves were milled (using an electric mill) to a fine and homogeneous powder, and stored in tightly closed and labeled containers until the time of extraction in a cool and dry place.

2.3 Preparation of the extracts

The dried powdered leaves were extracted into three extracts. The cold aqueous extract was obtained by suspending 10 g of the powder in 1000 mL of distilled water and stirring the suspension with a magnetic stirrer for 24 h at room temperature and later the suspension was filtered through Whatman No. 1 filter paper and the filtered solution was freeze dried (lyophilized). In the case of the hot aqueous extract, 10 g of powder was added to 200 mL of boiling distilled water, homogenized and left to stand for 30 min, after which it was separated by straining through gauze, clarified by centrifugation and concentrated using a rotary evaporator, at a temperature not exceeding 40 °C, to minimize thermal degradation, and stored at −20 °C. 10 g of the powder was suspended in 200 mL of 70% (v/v) ethanol and shaken for 24 h, filtered through sterile gauze, then through filter paper to obtain a clear, sediment free solution and then lyophilized to get the ethanolic extract. Moderately polar phenolics and less polar terpenoid constituents of leaf were recovered using aqueous ethanol as solvent [1] [8] [30]. All dry extracts were kept in tightly closed vials until required.

2.4 Extraction yield and qualitative phytochemical screening

The percentage yield of each extract was determined gravimetrically, using the following formula: mass of dry extract/mass of dry leaf powder × 100. The extracts were subsequently tested for their major classes of secondary metabolites by the following standard qualitative tests: ferric chloride test for tannins and total phenolics, alkaline reagent and Shinoda tests for flavonoids, froth test for saponins, Keller-Kiliani test for glycosides, Salkowski test for terpenoids and volatile oils, acetone-water test for resins, Mayer's reagent and Wagner's reagent for alkaloids. Following the general procedures of Harborne [30] the presence or absence of each class was recorded semi-quantitatively (absent to abundant) according to the intensity of the characteristic reaction.

2.5 Preparation of test concentrations

Each lyophilized (or rotary-evaporated) dry extract was reconstituted in its own sterile solventdistilled water for the cold and hot aqueous extracts and 70% (v/v) ethanol for the ethanolic extractto give a five-point concentration series of 12, 25, 50, 120 and 240 mg of dry extract per mL, corresponding to 1.2, 2.5, 5, 12 and 24% w/v (1% w/v = 1 g of dry extract per 100 mL of solvent = 10 mg/mL). These concentrations refer to the mass of dry extract in the reconstituted solution, not to the mass of the original leaf powder. The exact mass of dry extract weighed for each 10 mL of solvent is given in Appendix A (Table A3). All solutions were freshly prepared under aseptic conditions immediately before testing and were homogeneous and free of visible particles.

2.6 Test microorganisms and inoculum preparation

ISSN 2072-3857

Clinical isolates of E. coli and P. aeruginosa were acquired from an approved diagnostic laboratory. Each isolate was checked for its colonial morphology on selective and differential media, and for routine biochemical tests typical of the two species (E. coli: oxidase-negative, growth at 42 °C, indole- and methyl-red-positive, citrate-negative; P. aeruginosa: oxidase-positive, growth at 42 °C, indole- and methyl-red-negative, citrate-positive). All isolates were revived in nutrient broth (37 °C, 24 h) and a standardized suspension was prepared at turbidity equivalent to 0.5 McFarland standard, which corresponds to about 1×10^8 colony-forming units per ml (CFU/mL), according to the internationally recognised guidelines for susceptibility testing [25, 28]. The standardized suspension was used within 15 min of adjustment.

2.7 Agar well-diffusion assay

The antibacterial activity was determined using the agar well-diffusion method according to the literature [25]. Mueller-Hinton agar (MHA) was prepared, sterilized by autoclaving (121 °C, 15 min), cooled to approximately 45-50 °C and poured into sterile Petri dishes to a uniform depth of about 4 mm. A confluent lawn of bacteria was obtained by inoculating the surface of each plate with the standardized bacterial suspension using a sterile swab and the bacteria were allowed to absorb. Using a sterile cork borer, wells were bored into the agar (4-5 wells per plate) and 1.0 mL of the extract (at the required concentration) was added to each well. Plates were allowed to stand at room temperature for a brief pre-diffusion time, followed by incubation for 24 h at 37 °C. Each combination of extract-concentration-organism was replicated three times and the entire process was performed aseptically in the vicinity of a flame.

2.8 Controls, measurement and interpretation of activity

Sterile distilled water used as a negative (blank) control, and gentamicin (10 µg) and ciprofloxacin (5 µg) discs were used as positive reference standards to test both organisms, the latter to check the viability of the inocula, the quality of the media, and the incubation conditions, and to allow the absence of activity to be distinguished from a non-functional assay [24], [27], [28]. The diameter of any clear zone of inhibition surrounding each well (including the 6 mm well) was measured in millimetres (mm) after incubation with callipers. If no clearinggrowth was observed to the well margin, a treatment was recorded as 6 mm total diameter (0 mm net inhibition) and classified as inactive. Inhibition zones are typically categorized as weak (around 7-10 mm), moderate (11-15 mm) or strong (>15 mm) with the diameter of the well included.

2.9 Statistical analysis

All measurements were performed in three independent replicates and are presented as mean ± standard deviation (SD). In accordance with the experimental design (Section 2.1), the inhibition-zone data for the extracts were analysed as a 3 × 5 × 2 factorial arrangement by three-way analysis of variance (factorial ANOVA), with extract type (cold aqueous, hot aqueous, 70% ethanolic), concentration (five levels) and test organism (E. coli, P. aeruginosa) as fixed factors, and with all two-way and three-way interactions included in the model. Because every extract determination returned the same value (6.0 mm total diameter, i.e. 0 mm net inhibition), all cell means were identical and the residual (error) variance was zero; the sums of squares for all main effects and interactions were therefore also zero, F statistics were undefined, and the factorial analysis is reported descriptively in Section 3.5. The reference antibiotics (gentamicin and ciprofloxacin discs) were not part of the factorial arrangement and were compared with the extract and negative-control treatments in a separate analysis for each organism. Because

the zero variance of the extract and negative-control groups violates the homogeneity-of-variance assumption of parametric ANOVA, this comparison was performed with the non-parametric Kruskal–Wallis test across all treatment groups, followed by exact Mann–Whitney U tests comparing each reference antibiotic with the pooled extract and negative-control observations. Significance was set at $P < 0.05$. The Supplementary Dataset includes descriptive statistics and the full replicate-level data.

3. Results

3.1 Extraction yield and appearance of extracts

Coloured and homogeneous extracts were obtained by the three extraction methods, the yields are summarized in Table 1. The hot aqueous and 70% ethanolic procedures yielded highest recovery (19.2 and 20.6% w/w respectively) whereas, the cold aqueous maceration yielded the lowest recovery (13.4% w/w) due to higher solubilization of leaf components at high temperature and hydro-alcoholic solvent. The aqueous extracts were pale-to-medium brown while the ethanolic extract was a deeper greenish-brown, which was due to the co-extraction of chlorophyll and less polar terpenoids (Figure 1).

Table 1. Extraction yield and physical appearance of Myrtus communis leaf extracts (per 10 g dry leaf powder).

	Solvent	Yield (% w/w)	Appearance
Cold aqueous	Distilled water	13.4	Pale-to-medium brown
Hot aqueous	Boiling water	19.2	Medium brown
Ethanolic (70%)	70% ethanol	20.6	Deep greenish-brown

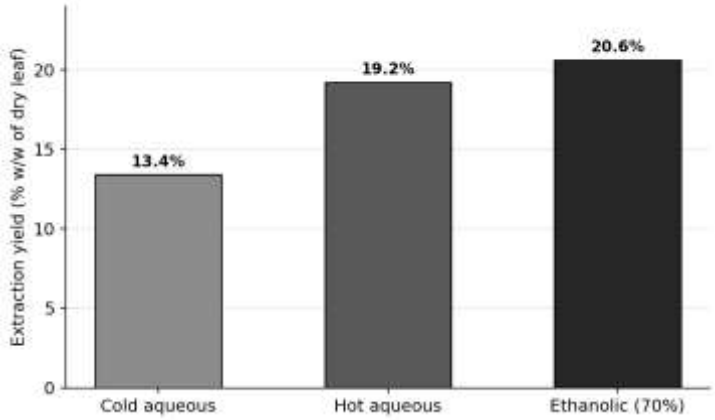


Figure 1. Extraction yield (% w/w of dry leaf) obtained by the three extraction procedures.

3.2 Phytochemical composition of the extracts

All three extracts were subjected to qualitative screening which indicated the presence of the main phenolic classes found in myrtle namely tannins, total phenolics, flavonoids and glycosides (Table 2). The aqueous extracts contained saponins, whereas terpenoid/volatile-oil constituents were detected in the hot aqueous (+) and, more abundantly, in the ethanolic (++) extract, and resins only in the ethanolic extract, which is in accordance with the higher lipophilic-solubilizing ability of the hydro-alcoholic extract. No alkaloids were found in any of the extracts. Importantly, therefore, the absence of inhibitory activity reported in Section 3.3 cannot be attributed to a failure to extract the

ISSN 2072-3857

putatively bioactive phenolic and terpenoid constituents, because these classes were detected in the extracts by qualitative screening.

Table 2. Qualitative phytochemical screening of Myrtus communis leaf extracts. Key: (−) absent; (+) present; (++) moderately abundant; (+++) abundant.

Constituent	Test used	Cold aqueous	Hot aqueous	Ethanolic (70%)
Tannins	Ferric chloride	++	++	++
Total phenolics	Ferric chloride / Folin	++	++	+++
Flavonoids	Alkaline / Shinoda	+	++	++
Saponins	Froth test	+	+	−
Glycosides	Keller-Kiliani	+	+	+
Terpenoids / volatile oil	Salkowski	−	+	++
Resins	Acetone-water	−	−	+
Alkaloids	Mayer / Wagner	−	−	−

Screening reflects the qualitative intensity of each characteristic colour/precipitation reaction; it is not a quantitative determination.

3.3 Antibacterial activity of the extracts

The three extracts of M. communis did not yield any zone of inhibition against E. coli and P. aeruginosa at any of the five concentrations tested. For all 90 extract determinations (3 extracts × 5 concentrations × 2 organisms × 3 replicates), the measured diameter was 6 mm (the well itself), with a mean total diameter of 6.0 ± 0.0 mm in each treatment group, equivalent to 0 mm net inhibition beyond the well edge. There was no clearing at the extract-agar interface and bacterial growth was extended up to the rim of each well. Complete replicate level results are shown in Table 3 (E. coli) and Table 4 (P. aeruginosa).

Table 3. Inhibition-zone diameters (mm) of Myrtus communis extracts against Escherichia coli full replicate data (R1-R3), mean ± SD, n = 3. I. = interpretation; (−) = inactive.

Extract	Conc. (%)	R1	R2	R3	Mean ± SD	I.
Cold aqueous	1.2	6	6	6	6.0 ± 0.0	−
	2.5	6	6	6	6.0 ± 0.0	−
	5	6	6	6	6.0 ± 0.0	−
	12	6	6	6	6.0 ± 0.0	−
	24	6	6	6	6.0 ± 0.0	−
Hot aqueous	1.2	6	6	6	6.0 ± 0.0	−
	2.5	6	6	6	6.0 ± 0.0	−
	5	6	6	6	6.0 ± 0.0	−
	12	6	6	6	6.0 ± 0.0	−
	24	6	6	6	6.0 ± 0.0	−
Ethanolic (70%)	1.2	6	6	6	6.0 ± 0.0	−
	2.5	6	6	6	6.0 ± 0.0	−
	5	6	6	6	6.0 ± 0.0	−
	12	6	6	6	6.0 ± 0.0	−

ISSN 2072-3857

Extract	Conc. (%)	R1	R2	R3	Mean ± SD	I.
	24	6	6	6	6.0 ± 0.0	−

Values include the 6 mm well diameter; 6 mm = growth up to the well edge (0 mm net inhibition).

Table 4. Inhibition-zone diameters (mm) of Myrtus communis extracts against Pseudomonas aeruginosa full replicate data (R1-R3), mean ± SD, n = 3. I. = interpretation; (−) = inactive.

Extract	Conc. (%)	R1	R2	R3	Mean ± SD	I.
Cold aqueous	1.2	6	6	6	6.0 ± 0.0	−
	2.5	6	6	6	6.0 ± 0.0	−
	5	6	6	6	6.0 ± 0.0	−
	12	6	6	6	6.0 ± 0.0	−
	24	6	6	6	6.0 ± 0.0	−
Hot aqueous	1.2	6	6	6	6.0 ± 0.0	−
	2.5	6	6	6	6.0 ± 0.0	−
	5	6	6	6	6.0 ± 0.0	−
	12	6	6	6	6.0 ± 0.0	−
	24	6	6	6	6.0 ± 0.0	−
Ethanolic (70%)	1.2	6	6	6	6.0 ± 0.0	−
	2.5	6	6	6	6.0 ± 0.0	−
	5	6	6	6	6.0 ± 0.0	−
	12	6	6	6	6.0 ± 0.0	−
	24	6	6	6	6.0 ± 0.0	−

Values include the 6 mm well diameter; 6 mm = growth up to the well edge (0 mm net inhibition).

3.4 Performance of controls and reference standards

The reference antibiotics showed clear reproducible zones of inhibitions against both organisms, indicating the bacterial inocula viability, and that the assay was working properly (Table 5). The mean zones of 20.3 ± 0.6 mm and 17.3 ± 0.6 mm were produced by gentamicin (10 µg) against E. coli and P. aeruginosa, respectively, whereas the mean zones of 24.3 ± 0.6 mm and 28.3 ± 0.6 mm were obtained by ciprofloxacin (5 µg) against the same pathogens, respectively. The negative control (sterile distilled water) did not show any inhibition for either organism. These data confirm that the lack of detected activity is not a result of methodological failure (inoculum viability, medium quality or incubation conditions), but reflects an absence of detectable activity of the myrtle extracts under the agar well-diffusion conditions used [24, 27].

Table 5. Reference-antibiotic and negative-control responses full replicate data (R1-R3), mean ± SD, n = 3.

Organism	Treatment	R1	R2	R3	Mean ± SD
Escherichia coli	Distilled water (neg.)	6	6	6	6.0 ± 0.0
Escherichia coli	**Gentamicin (10 µg)**	**20**	**21**	**20**	**20.3 ± 0.6**
Escherichia coli	**Ciprofloxacin (5 µg)**	**24**	**24**	**25**	**24.3 ± 0.6**
Pseudomonas aeruginosa	Distilled water (neg.)	6	6	6	6.0 ± 0.0

ISSN 2072-3857

Organism	Treatment	R1	R2	R3	Mean ± SD
Pseudomonas aeruginosa	**Gentamicin (10 µg)**	**17**	**18**	**17**	**17.3 ± 0.6**
Pseudomonas aeruginosa	**Ciprofloxacin (5 µg)**	**28**	**29**	**28**	**28.3 ± 0.6**

Reference-standard zones fall within the range expected for susceptible strains, verifying inoculum viability and medium performance.

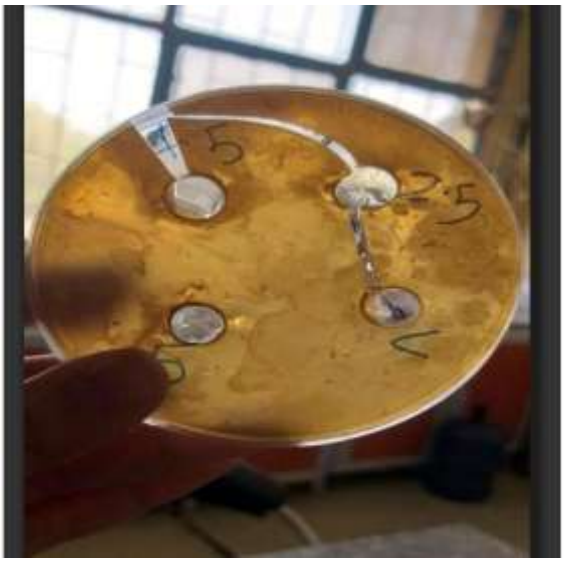


Figure 2. Agar well-diffusion plate on Mueller-Hinton agar showing myrtle extract loaded into bored wells (labelled by concentration) with no zone of inhibition around any well; bacterial growth extends to the well margins.

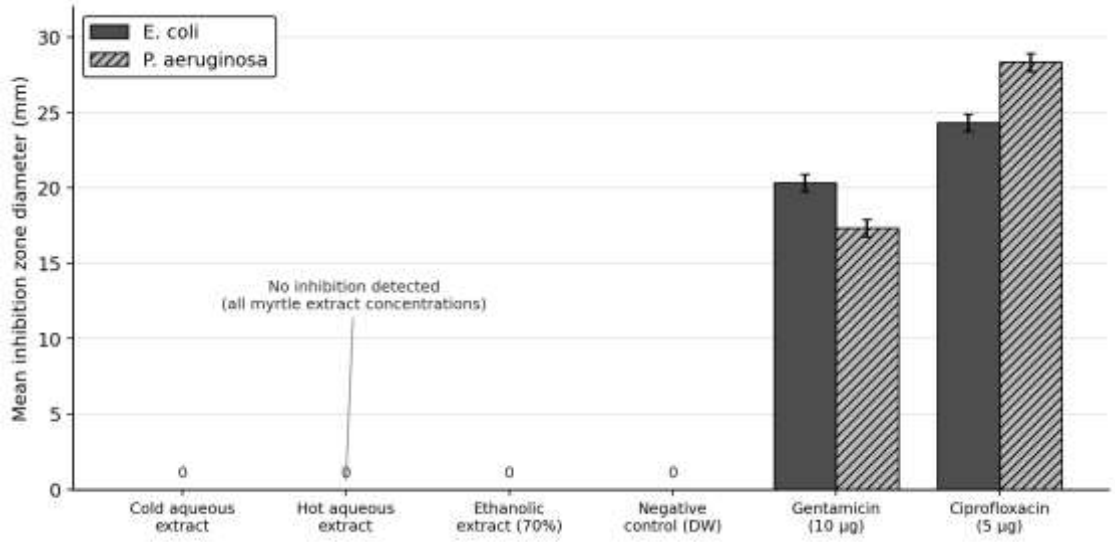


Figure 3. Mean net inhibition beyond the 6 mm well edge (mm ± SD) for each treatment against E. coli and P. aeruginosa. All myrtle extracts and the negative control gave 0 mm net inhibition, whereas gentamicin and ciprofloxacin produced clear zones (Kruskal–Wallis test, $P < 0.001$), validating the assay.

3.5 Statistical summary

All 90 extract determinations and all six negative-control determinations returned an

identical total diameter of 6 mm (0 mm net inhibition beyond the well). In the 3 × 5 × 2 factorial analysis, every cell mean was therefore 6.0 ± 0.0 mm; the sums of squares for extract type, concentration, test organism and all of their interactions were zero, the residual variance was zero and F statistics were undefined. No main effect of extract type, concentration or organism, and no interaction among them, could be detected, and no extract concentration differed from any other, from the other extracts or from the negative control for either organism. In the separate per-organism comparison with the reference standards, inhibition-zone diameter differed significantly among treatment groups (Kruskal–Wallis test, $P < 0.001$ for both organisms), an outcome attributable entirely to the antibiotic groups: gentamicin and ciprofloxacin each differed significantly from the pooled extract and negative-control observations (exact Mann–Whitney U test, $P < 0.001$), whereas all extract and negative-control groups shared the same value and could not be distinguished from one another. In practical terms, the myrtle extracts were statistically indistinguishable from the solvent blank and significantly less active than the antibiotic references.

4. Discussion

The principal finding of the study is negative: none of the three M. communis leaf extracts produced a detectable zone of inhibition against E. coli or P. aeruginosa in the agar well-diffusion assay, even at the highest concentration tested (240 mg/mL; 24% w/v). The result is not due to inoculum failure, defects in the media or to solvent interference since clear inhibition by gentamicin and ciprofloxacin was observed. The result therefore represents a valid negative finding for these extracts against these isolates under the diffusion-assay conditions used. It does not, however, exclude low-level activity that would be detectable only by dilution methods, because minimum inhibitory concentrations were not determined in this study. Notably, qualitative screening detected tannins, phenolics, flavonoids and glycosides in all extracts, and terpenoids in the hot aqueous and ethanolic extracts, so the outcome cannot be attributed to the failure to extract putatively active constituents, but rather to the bacterial physiology, extract chemistry and assay methodology as best understood by comparing with the existing literature.

A comparison of the results obtained is made with those of previously conducted studies.

The present findings were negative, but not out of the ordinary. They are compared with selected prior publications on myrtle antibacterial activity in Table 6. The most relevant study is that of Amensour and co-workers, which was conducted on methanol, ethanol and ethyl-acetate extracts of M. communis leaves and berries against a panel of food-borne and spoilage bacteria (E. coli K12 and Pseudomonas spp) by agar diffusion; none of the extracts was active against E. coli K12 and weak to moderate activity was reported for the extracts against Pseudomonas spp with a comparatively high minimum inhibitory concentration – in the order of 625 µg/mL – for the most active preparations [10]. Our uniform 0 mm net-inhibition result for E. coli (6 mm total well diameter) is in full agreement with their observation, and our negative diffusion zone result for P. aeruginosa is in agreement with their observation of having only weak, dilution-detectable activity; this would not normally be recorded in a diffusion zone assay.

Other studies do record activity, but the circumstances are different in ways that bring some harmony to the apparent conflict. Although inhibitory effects of leaf extracts of Javadian and colleagues were observed against antibiotic-resistant P. aeruginosa, the concentration of the extracts used to quantify the activity was mainly determined by the microdilution method (MIC) and was not determined by diffusion, and the effective concentrations were high [11]. The methanolic extracts or essential oil preparations that were used in the studies reporting antibacterial activity against E. coli or periodontal Gram-negative bacteria were richer in, or composed

ISSN 2072-3857

of, the lipophilic constituents, while our aqueous or hydroalcoholic extracts contained these only partly; the cited studies used diffusion and/or dilution methods [12, 13, 14]. The essential-oil and organic-solvent preparations characteristically are much more active against Gram-positive and acid-fast organisms with inhibition zones ranging from approximately 9-28 mm, and have a marked activity or ineffectiveness against Gram-negative organisms [8], [9], [15]. Considered as a body, the literature suggests that the antibacterial activity of myrtle is real, but highly dependent on the preparation, the type of the target and the type of assay used; our result falls on the heavily populated negative end of the spectrum of bacteria prepared by diffusion and tested against Gram-negative targets

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Table 6. Comparison of the present findings with previous studies on the antibacterial activity of Myrtus communis.

Study	Extract / part	Method	Organism(s)	Reported outcome	Ref.
Present study	Cold/hot aqueous & 70% ethanol leaf	Well diffusion, 1.2-24% w/v	E. coli; P. aeruginosa	No inhibition beyond well edge (6 mm total; 0 mm net), all conc.	
Amensour et al. (2010)	MeOH/EtOH/EtOAc leaf & berry	Agar diffusion	E. coli K12; Pseudomonas spp.	No activity vs E. coli K12; solvent-dependent activity vs Pseudomonas spp. (MIC ~625 µg/mL)	[10]
Javadian et al. (2019)	Ethanol & ethyl-acetate leaf	Diffusion + microdilution	Resistant P. aeruginosa	Activity reported (MIC determined)	[11]
Besufekad et al. (2017)	Leaf extracts	Agar diffusion	Several human pathogens	Activity vs multiple bacteria	[12]
Alem et al. (2008)	Crude myrtle preparation	Agar dilution	Common human pathogens	Some activity reported	[13]
Rahimvand et al. (2018)	Aqueous & methanolic leaf	Agar diffusion	Periodontal Gram-negatives	Activity reported	[14]
Zanetti et al. (2010)	Essential oil	Diffusion / dilution	Mycobacterium spp.	Antimycobacterial activity	[15]
EO / phenolic studies	Essential oil, phenolics	Diffusion / dilution	Mainly Gram-positive	Strong vs Gram-positive; variable/weak vs Gram-negative (≈9-28 mm)	[8],[9]

Reported values are as stated in the cited sources; direct numerical comparison is limited by differences in plant source, solvent, concentration units and assay format.

4.2 The Gram-negative outer-membrane barrier

The unique architecture of the Gram-negative cell envelope is the most significant biological factor. E. coli and P. aeruginosa have an outer membrane made up of an outer leaflet

consisting of lipopolysaccharide (LPS) which is stabilized by divalent cations and only perforated by size- and charge-selective porin channels [21, 22]. This outer membrane is a very efficient barrier for the permeation of many hydrophobic and amphipathic substances, such as a considerable portion of plant secondary metabolites [22] and [35]. Thus, the extracts from plants are generally more effective against Gram-positive bacteria than against Gram-negative bacteria under the same conditions [1], [8], [29]. The outer membrane is particularly resistant to penetration, and the low permeability is enhanced by efficient multidrug efflux systems, which accounts for the intrinsic resistance of P. aeruginosa which is well known [19, 20]. The same envelope may effectively prevent the action of conventional antibiotics and also inhibit the access of phytochemicals by these organisms, thus accounting for why extracts that are explicitly stated to contain phenolics and flavonoids were not effective against these organisms.

This interpretation is supported by experimental evidence to the effect that it is so. Mann and colleagues demonstrated that the outer membrane is the source of tolerance of P. aeruginosa to tea-tree essential oil, and that permeabilizing the outer membrane renders the organism susceptible again [23]. It is well established that Gram-negative bacteria become more susceptible to the action of hydrophobic antimicrobial agents by the use of agents that increase the permeability of their outer membrane, e.g., EDTA and polycationic compounds [35]. The fact that there is no such permeabilizer in the present assay is in agreement with the lack of activity and suggests that outer-membrane targeting for potentiation is a viable area of further study.

4.3 Extract chemistry and limitations of the diffusion assay

A second closely related factor is the chemistry of the extract vs. the assay format. The constituents of myrtleessential-oil that are generally attributed with antibacterial property are relatively non-polar such as terpenes (1,8-cineole, α-pinene, linalool) and the acylphloroglucinols (lipophilic) [8, 9]. These compounds are poorly soluble in the aqueous agar medium and even a compound that is active by itself may not form an inhibitory gradient around the well [24, 26]. In polar compounds, such as plant components, the method of agar diffusion is known to give false negatives and poor inter-laboratory reproducibility [24] and has been argued to be inherently unsuitable. Comparative methodological studies also indicate that the results of assays using the two methods, diffusion and dilution, may differ for the same extract; in general, lipid materials appear to be more sensitive when analyzed by the dilution method (broth or agar) [26, 25]. Therefore, it is reasonable to assume that the activity of the more lipophilic constituents in the extract, if present, was underestimated due to the use of diffusion format, as described in the cited research where the diffusion zone was very low or absent, but the dilution-detectable (MIC) was high in P. aeruginosa [10, 11].

Other factors such as extraction and dosing could also have played a role. Polar phenolics and tannins are preferentially extracted from the herbs by aqueous extraction; many terpenoid compounds are left in the aqueous extract, and those that are removed are not as effectively as by steam distillation or non-polar solvents [1], [31]. Concentrations of up to 24% (w/v) are high, but the actual amount of any one active ingredient reaching the well may be quite low, and losses due to diffusion may reduce the amount to the lawn even more [24]. Further reducing the likelihood of observing inhibition would be the use of robust strains of Gram-negative bacteria as clinical isolates, rather than more susceptible strains from the laboratory or strains of Gram-positive bacteria.The use of robust clinical strains of Gram-negative bacteria as opposed to more susceptible laboratory or Gram-

ISSN 2072-3857

positive strains would further reduce the likelihood of observing inhibition [19].

4.4 Implications for antimicrobial research

Translationally, the result is a welcome corrective. There is understandable interest and excitement in plant-based antimicrobials, but the drive for these agents needs to be balanced by thorough and transparent testing [2, 3]. Reporting negative results as in this study is critical to reduce publication bias that leads to an overestimation of the efficacy of phytochemicals in the literature and to help optimize the field towards preparations, targets and assay designs that are useful, informative. The consistent activity of the reference antibiotics in this same experiment highlights the need for standardized, validated methods to be used as a reference in assessing candidate natural products [25, 28]. Thus, for myrtle, the evidence indicates that its potential is not as a crude aqueous extract against Gram-negative bacteria, but rather is associated with higher concentrations of essential oil or purified phenolic extracts, and/or against Gram-positive bacteria and/or in combination approaches that disrupt the outer membrane barrier.

4.5 Limitations

There are a few cautions that need to be noted. First, only one assay using diffusion was performed, and broth or agar dilution assays, which are more suitable for constituents of lipophilicity, would give MIC values and may have given low level activity that was not detected by diffusion [24, 26]. Second, the extracts were only qualitatively screened and not quantitatively characterized (e.g. by GC-MS/HPLC) and therefore the absolute amount of individual active compounds is not known [9]. Third, a single geographic source and a limited concentration range were investigated, and no outer-membrane permeabilizer/antibiotic synergy testing was performed. 4. Only two species of Gram-negative bacteria were tested, with one isolate of each. These are limitations that establish an unambiguous research agenda of confirmatory research but do not undermine the internal validity of the current well-controlled observation.

5. Conclusion

The cold aqueous, hot aqueous and 70% ethanolic extracts of powdered M. communis leaves produced no detectable zone of inhibition against the tested clinical isolates of E. coli and P. aeruginosa at any concentration from 12 to 240 mg/mL (1.2–24% w/v) in the agar well-diffusion assay used in this study; all 90 extract determinations gave 6.0 ± 0.0 mm total diameter (0 mm net inhibition), identical to the sterile-water negative control. The validity of this negative result is supported by the reference antibiotics, which produced clear and reproducible inhibition zones against the same isolates (17.3–28.3 mm; $P < 0.001$ versus all extract and negative-control groups), and by the negative control, which produced none. Because only a diffusion format was used, no minimum inhibitory concentration was determined, and low-level activity detectable by broth or agar dilution cannot be excluded. Within these limits, the absence of detectable activity is most plausibly attributable to the Gram-negative outer-membrane permeability barrier, the poor diffusion of the largely non-polar constituents of myrtle through aqueous agar and the known limitations of diffusion assays for plant extracts, and it is consistent with several previous reportsmost closely that of Amensour et al. [10]of little or no diffusion-detectable activity of myrtle against these species.

These findings do not indicate that M. communis lacks antibacterial activity in general; rather, they show that the aqueous and 70% ethanolic leaf extracts, at the concentrations and in the assay format used here, were not active against these two Gram-negative isolates. Future studies should include (i) use of broth/agar dilution to determine minimum inhibitory concentrations;

(ii) chemical quantitation of extracts to correlate activity with plant material and solvent constituents; (iii) evaluation of a broader spectrum of solvents, plant sources and concentrations; and (iv) evaluation of outer-membrane permeabilizers and possible synergy with conventional antibiotics, which may give results not seen with the crude extracts alone.

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Appendix A. Experimental Materials, Reagents and Protocol

A.1 Instruments and equipment

Table A1. Instruments and equipment used in the study.

#	Instrument / item	Manufacturer	Country
1	Refrigerator	Marubeni	Japan
2	Water distiller	Ogawa	Japan
3	Incubator	Gallenkamp	England
4	Sensitive balance	Meter	Switzerland
5	pH meter	Radiometer	Denmark
6	Autoclave	Gallenkamp	England
7	Petri dishes	Local manufacture	Iraq
8	Micropipettes (variable volume)	Oxford	USA
9	Volumetric flasks	Glassco	India
10	Bunsen burner	Local manufacture	Iraq
11	Electric grinder / mill	Sony	Japan
12	Graduated cylinders	Glassco	India
13	Electric blender	Sony	Japan
14	Rotary evaporator	-	-
15	Lyophilizer (freeze-dryer)	-	-
16	Cork borer (6 mm)	Local manufacture	Iraq

A.2 Reagents and culture media

- Mueller-Hinton agar - antibacterial susceptibility testing (assay medium).
- Nutrient broth - revival and activation of bacterial isolates.
- Ethanol, 70% (v/v) - solvent for the ethanolic extract.
- Distilled water / sterile distilled water - solvent for aqueous extracts and negative control.
- Whatman No. 1 filter paper - clarification of extracts.
- Phytochemical reagents - ferric chloride, Mayer's/Wagner's, Keller-Kiliani, Salkowski, alkaline/Shinoda (qualitative screening).
- Gentamicin 10 µg and ciprofloxacin 5 µg discs - positive reference standards.

A.3 Concentration-preparation scheme

ISSN 2072-3857

Table A3. Preparation of the test concentration series for each extract. Each concentration was prepared by dissolving the stated mass of dry extract in 10 mL of sterile solvent. Concentrations are expressed as mg of dry extract per mL, with the equivalent percentage weight/volume (1% w/v = 1 g of dry extract per 100 mL = 10 mg/mL).

Final concentration (mg dry extract/mL)	Equivalent (% w/v)	Dry extract weighed (g)	Sterile solvent added (mL)
12	1.2	0.12	10
25	2.5	0.25	10
50	5	0.50	10
120	12	1.20	10
240	24	2.40	10

Solvent = sterile distilled water (cold and hot aqueous extracts) or 70% (v/v) ethanol (ethanolic extract). Values are nominal concentrations of dry (lyophilized or rotary-evaporated) extract in the reconstituted test solution; they do not refer to the mass of the original leaf powder. The five concentrations correspond to those reported as 1.2, 2.5, 5, 12 and 24% (w/v) in Tables 3 and 4. Each concentration was loaded at 1.0 mL per 6 mm well, with three replicate wells per concentration per organism.

A.4 Agar well-diffusion protocol (step-by-step)

1. Prepare and autoclave Mueller-Hinton agar (121 °C, 15 min); cool to ~45-50 °C and pour into sterile Petri dishes (~4 mm depth); allow to solidify.
2. Revive each isolate in nutrient broth (37 °C, 24 h); confirm identity by colonial morphology and biochemical tests.
3. Standardize the suspension to ~1 × 10^8 CFU/mL (0.5 McFarland).
4. Swab the agar surface to create a confluent bacterial lawn; allow to dry briefly.
5. Bore 6 mm wells with a flame-sterilized cork borer (4-5 wells per plate).
6. Dispense 1.0 mL of the test extract concentration into each well; reserve one well for the sterile-water negative control.
7. Include gentamicin (10 µg) and ciprofloxacin (5 µg) discs on parallel plates as positive reference standards.
8. Incubate plates at 37 °C for 24 h.
9. Measure the diameter of any inhibition zone (mm, including the 6 mm well) with calibrated callipers; record 6 mm total diameter (0 mm net inhibition) where growth reaches the well edge.
10. Repeat for three independent replicates per treatment; compute mean ± SD; analyse the extract data by three-way factorial ANOVA (extract type × concentration × organism) and compare the reference standards with the extract and negative-control groups by the Kruskal–Wallis test (Section 2.9).

A.5 Plant material and workflow

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Figure A1. Freshly collected Myrtus communis foliage prior to drying.



Figure A2. Air-dried myrtle leaves used to prepare the leaf powder.

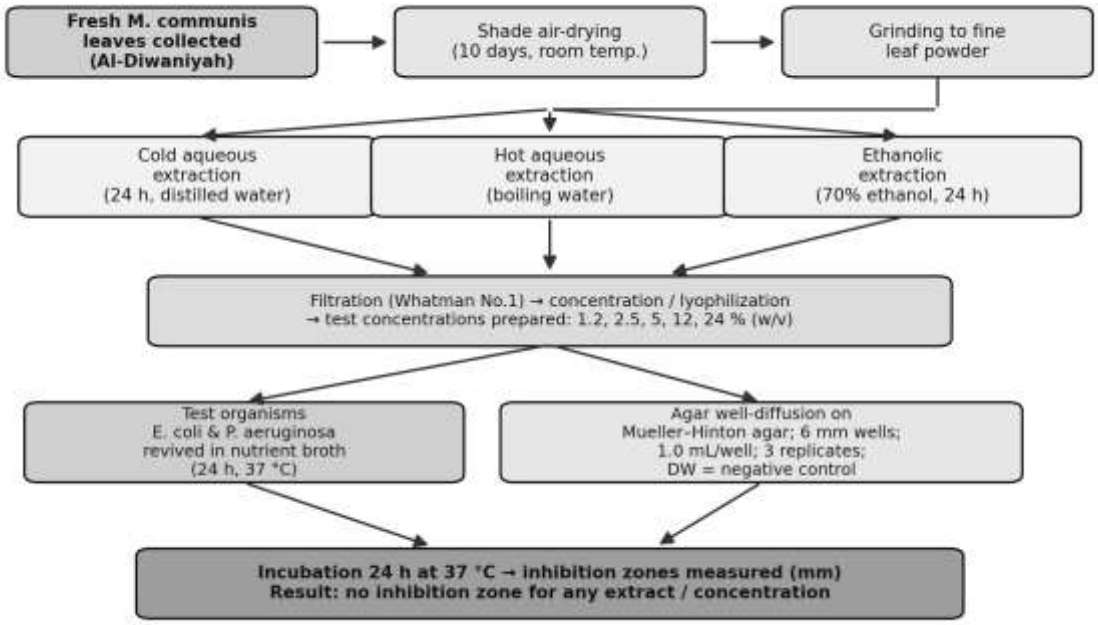


Figure A3. Overview of the experimental workflow, from plant collection and extraction to the agar well-diffusion assay and the null inhibition outcome.

ISSN 2072-3857