ATTAHADI MEDICAL JOURNAL
Journal homepage:
http://attahadi.edu.ly/journal/index.php/amj/index

Antibacterial Properties of *Alchemilla vulgaris* and *Euphorbia helioscopia*: Potential Natural Alternatives for the Treatment of Conjunctivitis

Abdelsalam Abubaker [1], Shamsi Saad[2*], Abdlrhman Alsonosi[3]

[1]*Department of Botany, Faculty of Science, Sebha University, Sebha, Libya*
[2]*Department of Medical Laboratory, Faculty of Medical Technology, Sebha University, Sebha, Libya*
[3]*Department of Microbiology, Faculty of Medicine, Sebha University, Sebha, Libya*

Keywords:
Alchemilla vulgaris, Euphorbia helioscopia. Conjunctivitis.

Received *12 July 25*
Accepted *17 Sept 25*
Published *22 Sept 25*

ABSTRACT

The emergence of antibiotic-resistant bacteria necessitates the exploration of alternative therapeutic agents. This study investigates the antibacterial potential of *Alchemilla vulgaris* and *Euphorbia helioscopia* extracts against bacterial strains associated with conjunctivitis, including Staphylococcus aureus, Pseudomonas aeruginosa, and Streptococcus spp. Plant extracts were prepared using water, ethanol, hexane, and chloroform as solvents, and their antibacterial efficacy was assessed via agar well diffusion and broth microdilution minimum inhibitory concentration (MIC) assays. Ethanol extracts demonstrated the highest antibacterial activity in agar diffusion, with inhibition zones up to 17 mm for E. helioscopia and 12 mm for A. vulgaris (against Streptococcus spp.). Chloroform extracts showed moderate activity (zones ranging from 9–19 mm across both plants), while hexane extracts exhibited lower activity. Water extracts displayed no antibacterial effects. MIC analysis revealed that the ethanol extracts had the lowest MIC values (approximately 280 mg/mL) among all extracts, notably against S. aureus (for A. vulgaris) and P. aeruginosa (for E. helioscopia). All other solvent extracts showed higher MICs (≥550–750 mg/mL). Statistical analysis confirmed significant differences in the antibacterial activity of the extracts compared to the control antibiotic gentamicin (30 µg) ($p < 0.05$). These findings highlight the potential of *Alchemilla vulgaris* and *Euphorbia helioscopia* as sources of natural antibacterial agents. Future studies should focus on isolating bioactive compounds and evaluating their pharmacological properties to develop alternative treatments for ocular bacterial infections.

Citation info. Abubaker A, Saad S, Alsonosi A. Antibacterial Properties of *Alchemilla vulgaris* and *Euphorbia helioscopia*: Potential Natural Alternatives for the Treatment of Conjunctivitis. Attahadi Med J. 2025;2(3):324-333. https://doi.org/10.69667/10.69667/amj.25323

INTRODUCTION

Antibiotic resistance has undermined the effectiveness of first-line therapies for common bacterial infections, including ocular diseases such as conjunctivitis. Although empirical topical antibiotics remain standard care, resistance among typical conjunctivitis pathogens—Staphylococcus aureus, Streptococcus spp., and Gram-negative bacilli such as Pseudomonas aeruginosa—is increasingly reported and can raise the risk of treatment failure, prolonged symptoms, and community spread [1,2,3,4].

Medicinal plants provide a chemically diverse source of antimicrobial scaffolds that can complement or potentiate conventional antibiotics. *Alchemilla vulgaris* (lady's mantle, Rosaceae) contains tannins, flavonoids, and triterpenoids with reported anti-inflammatory and antibacterial effects [5,6]. *Euphorbia helioscopia* (sun spurge, Euphorbiaceae) is rich in diterpenes and phenolics and has shown in vitro antibacterial activity against Gram-positive organisms, including methicillin-resistant S. aureus (MRSA). Mechanistically, polyphenols and flavonoids can disrupt microbial membranes, inhibit enzyme and nucleic acid targets, and impair biofilms—properties relevant to ocular pathogens [6].

Despite accumulating evidence of plant-derived antimicrobials, data directly targeting ocular pathogens and conjunctivitis-related isolates remain scarce. Moreover, solvent choice strongly influences the recovery of active phytochemicals: polar solvents (e.g., aqueous ethanol) often yield higher phenolic and flavonoid content with stronger antimicrobial activity than non-polar solvents, yet head-to-head comparisons for A. vulgaris and E. helioscopia are limited [7,8,9].

This study aimed to evaluate the antibacterial activity of water, ethanol, hexane, and chloroform extracts from A. vulgaris and E. helioscopia against conjunctivitis-associated bacteria (S. aureus, Streptococcus spp., and P. aeruginosa), using agar well diffusion and broth microdilution MIC assays.

***Corresponding E-mail addresses**: Sha.saad@sebhau.edu.ly

This is an open-access article under the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0).

ATTAHADI MEDICAL JOURNAL
Journal homepage:
http://attahadi.edu.ly/journal/index.php/amj/index

MATERIALS AND METHODS

Study design and ethics

This experimental study assessed crude plant extracts against clinical ocular isolates using agar well diffusion and broth microdilution MIC assays, following Clinical and Laboratory Standards Institute (CLSI) and EUCAST guidelines where applicable [10]. Clinical sampling and data handling complied with the Declaration of Helsinki. Written informed consent was obtained from all participants.

Plant material and authentication

Fresh aerial parts (leaves and stems) of *Alchemilla vulgaris* and *Euphorbia helioscopia* were collected from Gharyan, Libya, in Fall 2024. Plant species were authenticated by a botanist, and voucher specimens were deposited at the Herbarium of the Botany Department, Faculty of Science, Sebha University (Sebha, Libya).

Reagents and media

Ethanol (96% v/v, analytical grade), n-hexane, and chloroform were obtained from Sigma-Aldrich (St. Louis, MO, USA). Distilled water was produced in-house. Culture media included Mueller–Hinton agar (MHA) and cation-adjusted Mueller–Hinton broth (CAMHB) for susceptibility testing, Blood agar (5% defibrinated sheep blood) and Chocolate agar for primary ocular isolation, and Mannitol Salt Agar, MacConkey agar, and Cetrimide agar for selective isolation of specific pathogens; all media were purchased from Oxoid (Basingstoke, UK). Gentamicin antibiotic discs (30 µg) were from Oxoid. Syringe filters (0.22 µm pore) were obtained from Millipore (Burlington, MA, USA). A calibrated digital caliper (Mitutoyo, Japan) was used for measuring inhibition zones. API identification panel kits (bioMérieux, Marcy-l'Étoile, France) were used for biochemical confirmation of bacterial isolates.

Preparation of plant extracts

The collected plant materials were rinsed with water, shade-dried at ≤40 °C to constant weight, and milled to a fine powder (~40 mesh particle size). For each solvent, 100 g of dried powder was extracted by Soxhlet apparatus with 1.0 L of solvent (water, ethanol, hexane, or chloroform) for 6–8 h, until the siphon runs were nearly colorless. The resulting filtrates were concentrated under reduced pressure at ≤40 °C using a rotary evaporator (Büchi Rotavapor R-300, Flawil, Switzerland), then dried to constant mass and stored in amber vials at –20 °C until use. Extraction yield (%) was calculated as the mass of dried extract divided by the mass of starting plant powder × 100. (Table 1) summarizes the extraction yields for each solvent and plant.

Sterile stock solutions of each dried extract were prepared (approximately 400 mg/mL) using a minimal amount of organic solvent (ethanol or dimethyl sulfoxide, such that solvent content was ≤1% v/v in final assays). The stock solutions were filter-sterilized through 0.22 µm filters and stored at 4 °C for up to 72 h (or at –20 °C for longer storage) until testing. This extraction procedure ensured an exhaustive recovery of phytochemicals: in general, ethanol (polar) preferentially extracts phenolics and flavonoids, hexane (non-polar) retrieves lipophilic constituents such as certain terpenoids, and chloroform (mid-polarity) yields intermediate-polarity compounds potentially relevant to antibacterial activity.

Clinical isolates and reference strains

Eye swab specimens were collected from patients with clinical conjunctivitis at Sebha Medical Center (Sebha, Libya) between January and March 2024. Inclusion criteria were acute conjunctivitis with clinical signs suggestive of bacterial infection; exclusion criteria were recent antibiotic use (≤ 72 h) or a non-bacterial diagnosis. Swabs were transported in Amies transport medium at 2–8 °C and cultured within 4 hours of collection.

For primary culture, samples were streaked onto Blood agar and Chocolate agar (incubated at 35 ± 2 °C; Chocolate agar in 5% CO_2 atmosphere), MacConkey agar (35 ± 2 °C, ambient air) for Gram-negative bacteria, Mannitol Salt Agar for staphylococci, and Cetrimide agar for presumptive Pseudomonas aeruginosa. After 18–24 h incubation, isolates were examined for colony morphology (including hemolysis on blood agar, pigment production, odor), Gram stained, and subjected to preliminary biochemical tests: catalase and coagulase tests for Staphylococcus aureus; oxidase test and assessment of non-fermentative metabolism (no acid from glucose) for P. aeruginosa; and for Streptococcus spp., catalase negativity followed by specific tests, Final species identification of isolates was confirmed using API biochemical identification strips appropriate to the organism (bioMérieux).

For antibacterial assays, standardized inocula were prepared by suspending fresh (18–24 h) bacterial cultures in sterile 0.85% saline to achieve a turbidity of 0.5 McFarland (~1–2 × 10^8 CFU/mL). For each experiment, this suspension was diluted in growth medium to the required cell density (typically ~5 × 10^5 CFU/mL for MIC assays). Quality control (QC) reference strains used in susceptibility testing included QC strains (E. coli AC124, S. aureus DF256, and P. aeruginosa AC110), which were obtained from the laboratory culture collection and tested in parallel to validate the methods.

ATTAHADI MEDICAL JOURNAL
Journal homepage:
http://attahadi.edu.ly/journal/index.php/amj/index

Agar well diffusion assay

Antibacterial screening by agar well diffusion was performed on MHA plates (for Streptococcus spp., MHA supplemented with 5% sheep blood). Each plate was swabbed evenly with a bacterial suspension (0.5 McFarland standard) to create a confluent lawn. Wells of 6 mm diameter were aseptically bored into the agar and filled with 100 µL of the extract solution. Each well contained a standardized amount of extract (approximately 100 mg of dried extract per well, corresponding to a 1000 mg/mL stock concentration). Controls included a positive control antibiotic (gentamicin 30 µg disc placed on the same plate) and a negative control (100 µL of solvent vehicle with ≤1% v/v ethanol or DMSO, which produced no inhibition zone). Plates were incubated at 35 ± 2 °C for 18–24 h. Plates for S. pneumoniae (or other streptococci) were incubated in a 5% CO_2 atmosphere, whereas plates for S. aureus and P. aeruginosa were incubated in ambient air. After incubation, the diameters of inhibition zones were measured in millimeters using the digital caliper, across the center of each well (for wells) or across the disc diameter (for gentamicin), excluding the 6 mm well diameter. All tests were performed in triplicate, independent experiments.

Minimum inhibitory concentration (MIC) by broth microdilution

MICs were determined using the broth microdilution method in 96-well microplates [8]. The assays were carried out in CAMHB for S. aureus and P. aeruginosa; for Streptococcus spp., CAMHB was supplemented with 2–5% lysed horse blood to support growth. Two-fold serial dilutions of each extract were prepared in the broth to achieve a concentration range extending up to a high of 750 mg/mL. Specifically, each well contained 100 µL of an extract dilution and 100 µL of bacterial suspension, yielding a final inoculum of ~5 × 10^5 CFU/mL in each well. The final extract concentrations in the wells ranged, for example, from about 4 mg/mL to 750 mg/mL across the dilution series. Solvent controls (broth with equivalent ≤1% v/v DMSO or ethanol) and a growth control (bacteria in broth without extract) were included on each plate, as well as a sterility control (broth only). The microplates were incubated at 35 ± 2 °C for 18–24 h (in ambient air for S. aureus and P. aeruginosa, and 5% CO_2 for Streptococcus as above). The MIC was defined as the lowest extract concentration that completely inhibited visible growth (no turbidity) in the well. In cases of ambiguity, 0.01% resazurin dye was added to wells, and color change (blue indicating no growth, pink indicating growth) was observed for confirmation. All MIC determinations were performed in triplicate.

Data handling and statistical analysis

The primary outcome measures were the diameters of inhibition zones (mm) in the agar diffusion assay, and the MIC values (mg/mL) from the broth microdilution assay were considered secondary outcomes. Quantitative data are reported as mean ± standard deviation (SD) for triplicate experiments. Data were tested for normality (Shapiro–Wilk test) and homogeneity of variances (Levene's test). Depending on the experimental design, either a one-way analysis of variance (ANOVA) was used (e.g., to compare the effects of different solvent extracts within the same plant species) or a two-way ANOVA (to compare factors of plant species and solvent). Post-hoc comparisons were conducted with appropriate tests (Dunnett's test when comparing each extract to the gentamicin control, or Tukey's test for all pairwise comparisons in a two-way ANOVA). A significance level of $\alpha = 0.05$ was applied. Calculated p-values are reported to three decimal places when applicable. Statistical analyses were performed using GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA).

RESULTS

Extraction yields (percentage of dry extract obtained from dried plant material) varied markedly with solvent polarity and between the two plant species. *Alchemilla vulgaris* yielded approximately 1.0% with water extraction, 6.0% with ethanol, 2.0% with hexane, and 8.0% with chloroform. *Euphorbia helioscopia* yielded about 1.1% with water, 5.5% with ethanol, 6.2% with hexane, and 2.2% with chloroform. These yield profiles suggest that A. vulgaris provided a greater extractable mass in mid-polar solvent (chloroform), whereas E. helioscopia gave a higher yield in non-polar solvent (hexane), reflecting differences in phytochemical composition between the two plants. (Table 1) summarizes the extraction yields for each solvent and plant.

Table 1. Extraction yields (% w/w of dried plant material) of each solvent extract for Alchemilla vulgaris and Euphorbia helioscopia.

Plant species	Water extract (%)	Ethanol extract (%)	Hexane extract (%)	Chloroform extract (%)
Alchemilla vulgaris	1.0	6.0	2.0	8.0
Euphorbia helioscopia	1.1	5.5	6.2	2.2

Bacterial Isolates Recovered

In total, 20 culture-positive conjunctivitis samples were analyzed. Staphylococcus aureus was the most frequent isolate (identified in 9 of 20 cases, 45%), followed by Streptococcus spp. (7 cases, 35%) and Pseudomonas aeruginosa (4 cases, 20%). This distribution is consistent with the general

ATTAHADI MEDICAL JOURNAL
Journal homepage:
http://attahadi.edu.ly/journal/index.php/amj/index

epidemiology of acute bacterial conjunctivitis, where Gram-positive pathogens like Staphylococcus and Streptococcus usually predominate, and Gram-negative bacteria such as P. aeruginosa represent a smaller proportion of isolates. All clinical isolates were confirmed by microbiological and biochemical methods as described, and their identities align with common causative agents of conjunctivitis [1,2]. The confirmed S. aureus and P. aeruginosa isolates were used for subsequent antimicrobial testing, along with a representative Streptococcus pneumoniae among the streptococcal isolates.

Agar Well Diffusion Assay

In the agar diffusion assay, the diameter of inhibition zones varied with both the plant extract and the solvent used, as well as with the bacterial species tested. Notably, no inhibition was observed with any water extract from either plant (0 mm zones for all water extract tests), underscoring the lack of antibacterial activity in the aqueous extracts at the tested concentration. In contrast, the positive control (gentamicin 30 µg) produced the largest clear zones against all bacteria (mean ~24 mm, ranging 22–27 mm, depending on the organism), confirming the susceptibility of the isolates and validity of the assay.

For *Euphorbia helioscopia*, the ethanol extract exhibited the strongest antibacterial activity among the plant's extracts, yielding inhibition zones of approximately 17 mm against S. aureus, 14 mm against P. aeruginosa, and 17 mm against Streptococcus spp. (Table 2). The E. helioscopia hexane extract showed only minimal inhibition (zones ~3–10 mm), while the chloroform extract produced intermediate effects (zones ~9–19 mm, with the largest zone of 19 mm against P. aeruginosa).

For *Alchemilla vulgaris*, a somewhat different pattern was observed. The ethanol extract inhibited S. aureus and P. aeruginosa with zones of 10 mm, and Streptococcus spp. with 12 mm. The hexane extract zones were modest (8–10 mm). Notably, the chloroform extract of A. vulgaris was relatively more effective, producing the largest inhibition zone of 20 mm against P. aeruginosa, along with 9 mm against S. aureus and 17 mm against Streptococcus spp. (Table 3). Neither plant's water extract produced any zone of inhibition. Gentamicin controls for each organism ranged from 22 mm (S. aureus and Streptococcus) up to 27 mm (P. aeruginosa).

Overall, the ethanol extracts of both plants showed superior activity against the Gram-positive bacteria, whereas the A. vulgaris chloroform extract stood out for activity against the Gram-negative P. aeruginosa. The hexane extracts were the least active of the organic solvents. These differences were reflected in the statistical analysis: for each plant, there were highly significant differences in mean inhibition zone between the different extract types (one-way ANOVA for E. helioscopia extracts: $F = 20.21$, $p < 0.001$; for A. vulgaris: $F = 26.31$, $p < 0.001$). Post-hoc comparisons confirmed that the ethanol and chloroform extracts produced significantly larger zones than the negative control (vehicle) and significantly different zones compared to each other in many cases ($p < 0.05$), whereas the water extracts differed from all other groups only by having no effect. The gentamicin control produced significantly larger zones than any plant extract ($p < 0.001$ vs all extracts). (Tables 2 and 3) present the detailed zone of inhibition results for each plant extract and organism.

Table 2. Agar well diffusion inhibition zones (mean diameter in mm) for Euphorbia helioscopia extracts against conjunctivitis-associated bacteria. (Each well contained 100 µL extract at ~1000 mg/mL. Values are means of triplicates, rounded to the nearest millimeter.)

Bacterial isolate	Water (no extract)	Ethanol extract	Hexane extract	Chloroform extract	Gentamicin 30 µg (control)
Staphylococcus aureus	0 mm	17 mm	3 mm	9 mm	22 mm
Pseudomonas aeruginosa	0 mm	14 mm	9 mm	19 mm	27 mm
Streptococcus spp.	0 mm	17 mm	10 mm	9 mm	22 mm

Table 3. Agar well diffusion inhibition zones (mean diameter in mm) for Alchemilla vulgaris extracts against conjunctivitis-associated bacteria.

Bacterial isolate	Water (no extract)	Ethanol extract	Hexane extract	Chloroform extract	Gentamicin 30 µg (control)
Staphylococcus aureus	0 mm	10 mm	9 mm	9 mm	22 mm
Pseudomonas aeruginosa	0 mm	10 mm	8 mm	20 mm	27 mm
Streptococcus spp.	0 mm	12 mm	10 mm	17 mm	22 mm

(Note: "0 mm" indicates no detectable inhibition zone beyond the 6 mm well diameter.)

ATTAHADI MEDICAL JOURNAL
Journal homepage:
http://attahadi.edu.ly/journal/index.php/amj/index

Minimum Inhibitory Concentrations (MICs)

The MIC values of each extract against the tested bacteria are presented in Tables 4&5. In general, the MIC results corroborated the diffusion assay findings, but also highlighted the relatively high concentrations of crude extracts required to inhibit bacterial growth in broth culture.

For *Alchemilla vulgaris* (Table 4), the ethanol extract had the lowest MIC, achieving inhibition of all three test organisms at 280 mg/mL. In contrast, the hexane and chloroform extracts of A. vulgaris required much higher concentrations to inhibit growth (MIC = 750 mg/mL for each bacterial strain). The water extract showed no inhibitory effect at the maximum concentration tested (no growth inhibition even at 750 mg/mL, denoted as ">750"). These results indicate that the ethanol-soluble components of A. vulgaris are the most potent in terms of antibacterial activity, whereas the hexane- and chloroform-soluble fractions, despite showing some zones in the agar test, were far less effective in broth (requiring the highest concentrations to achieve inhibition).

For *Euphorbia helioscopia* (Table 5), a somewhat different MIC pattern was observed for the Gram-positive vs. Gram-negative targets. The E. helioscopia ethanol and hexane extracts both showed MICs of 750 mg/mL against S. aureus and Streptococcus spp., suggesting only weak inhibition at the highest concentrations. However, against P. aeruginosa, the ethanol extract of E. helioscopia was notably more potent (MIC = 280 mg/mL), aligning with the relatively large agar inhibition zone observed for P. aeruginosa. The chloroform extract of E. helioscopia showed an interesting selectivity: it had an MIC of 750 mg/mL for S. aureus and P. aeruginosa, but was slightly more effective against Streptococcus spp. (MIC = 550 mg/mL). As with A. vulgaris, the water extract of E. helioscopia had no detectable activity (no inhibition at ≤750 mg/mL).

In summary, the lowest MIC recorded in this study was 280 mg/mL (for the ethanol extracts of both plants against their most susceptible organisms: S. aureus for A. vulgaris and P. aeruginosa for E. helioscopia). While these MIC values are high in absolute terms (hundreds of mg/mL), they provide a quantitative confirmation that the crude ethanol extracts contain the primary antibacterial constituents. The much higher MICs (550–750 mg/mL) required by the hexane and chloroform extracts reflect their lower potency, despite some showing moderate zones in the diffusion assay. It is also evident that P. aeruginosa was more difficult to inhibit in broth for most extracts, except the E. helioscopia ethanol extract, consistent with P. aeruginosa's greater intrinsic resistance. No MIC could be determined for water extracts up to the solubility limit of the assay, reinforcing that the water-soluble fractions lacked antibacterial activity.

Table 4. Minimum inhibitory concentrations (MICs) of Alchemilla vulgaris solvent extracts against conjunctivitis pathogens (values in mg/mL).

Bacterial isolate	Ethanol extract	Hexane extract	Chloroform extract	Water extract
Staphylococcus aureus	280	750	750	>750
Streptococcus spp.	280	750	750	>750
Pseudomonas aeruginosa	280	750	750	>750

Table 5. Minimum inhibitory concentrations of Euphorbia helioscopia extracts against conjunctivitis pathogens (mg/mL).

Bacterial isolate	Ethanol extract	Hexane extract	Chloroform extract	Water extract
Staphylococcus aureus	750	750	750	>750
Streptococcus spp.	750	750	550	>750
Pseudomonas aeruginosa	280	750	750	>750

(Note: ">750" indicates no inhibition observed at the maximum tested concentration of 750 mg/mL, i.e., MIC exceeds this value.)

Statistical analysis of assays

The agar diffusion results were subjected to analysis of variance as described. For each plant, there was a statistically significant effect of extract type on the size of the inhibition zone ($p < 0.001$), confirming that the choice of solvent extract significantly altered antibacterial performance. In post-hoc analyses, the ethanol extracts of both plants produced significantly larger zones against the Gram-positive bacteria compared to the other extracts ($p < 0.01$ for E. helioscopia ethanol vs its hexane or water extracts, for example). Similarly, A. vulgaris chloroform extract produced a significantly larger zone against P. aeruginosa than the ethanol extract of A. vulgaris ($p < 0.05$), highlighting the species-specific effect noted above. All extract treatments yielded zones significantly smaller than gentamicin ($p < 0.001$).

For the MIC data, statistical comparison between extracts was descriptive due to the limited number of concentration points yielding total inhibition. However, the ethanol extracts achieved inhibition at

concentrations significantly lower than the other extracts, reinforcing the qualitative rankings seen in the diffusion test.

DISCUSSION

Importance of plant phytochemicals and solvent effects: The two medicinal plants investigated in this study, A. vulgaris and E. helioscopia, are known to contain distinct profiles of bioactive constituents, which influenced both the extraction yields and the antibacterial effects observed. Alchemilla species are rich in polyphenolic compounds such as tannins and flavonoids [11,12]. These compounds are predominantly polar and water-soluble (especially as glycosides or polyphenolic acids), so it was expected that polar solvents like ethanol would extract them efficiently. Indeed, A. vulgaris showed a higher yield with ethanol than with hexane, and its ethanol extract demonstrated broad antibacterial activity, consistent with enrichment of phenolics. Euphorbia species (including E. helioscopia) contain abundant diterpenoid esters, triterpenes, and other lipophilic constituents [13]. These non-polar or mid-polarity compounds were better extracted by hexane and chloroform, explaining why E. Helioscopia gave a relatively high hexane extract yield compared to its ethanol yield. However, it is important to note that extraction yield does not directly equate to antibacterial potency. Non-bioactive matrix substances (e.g., chlorophylls, waxes) can contribute to high yield in non-polar extracts without adding antimicrobial effect [14]. In this study, the ethanol extracts, despite moderate yields, showed the strongest antibacterial outcomes, implying that they contained more potent antimicrobial compounds per unit mass. The divergent yield profiles observed (higher chloroform yield for A. vulgaris, higher hexane yield for E. helioscopia) are thus reflective of each plant's phytochemistry, but the biological results underscore that the composition of the extract is more critical than the quantity of crude extract obtained.

Bacterial isolates and comparison with literature: The clinical isolates used in this research (S. aureus, Streptococcus pneumoniae among Streptococcus spp., and P. aeruginosa) represent the primary bacterial pathogens in conjunctivitis. As observed, Gram-positive cocci (particularly S. aureus) constituted most of our isolates, with Streptococcus spp. also common, while Gram-negative Pseudomonas was less frequent. This distribution aligns with reports from other epidemiological studies of bacterial conjunctivitis, which indicate that Staphylococcus species are often the most prevalent pathogens, followed by Streptococcus (especially S. pneumoniae in some populations), whereas Pseudomonas and other Gram-negatives are encountered more rarely [1,2]. Our findings conform to this pattern, reinforcing that the test panel of bacteria in this study is clinically relevant. Additionally, all isolates were confirmed to be susceptible to gentamicin in vitro, a standard ophthalmic antibiotic, although resistance to such agents has been rising globally [15]. By using actual patient isolates rather than reference strains alone, our study provides insight into how these plant extracts perform against real-world conjunctivitis pathogens, which is an important strength.

Agar diffusion assay results – solvent and organism effects: The agar well diffusion tests demonstrated two main trends: (1) Solvent-dependent efficacy – extracts obtained with different solvents showed markedly different antibacterial activities, and (2) Organism-specific susceptibility – the Gram-negative bacterium P. aeruginosa was generally less susceptible to the plant extracts than the Gram-positive bacteria (S. aureus and Streptococcus). These trends agree with general findings in natural product research: the choice of solvent can enrich for different sets of compounds with varying bioactivities [4], and Gram-negative bacteria often exhibit higher intrinsic resistance to antimicrobials due to permeability barriers [16,17].

In our study, the ethanol extracts of both plants showed the largest inhibition zones overall, particularly against the Gram-positive organisms. This suggests that ethanol successfully extracted antibacterial agents (likely polyphenols/flavonoids and related compounds) that are effective against S. aureus and streptococci. Flavonoids and tannins can exert multiple antibacterial mechanisms – including disruption of cell membranes, inhibition of extracellular enzymes and toxins, and interference with nutrient uptake or nucleic acid synthesis – which tend to be especially effective against Gram-positive bacteria whose cell envelopes are less complex [17,18]. Our finding that E. helioscopia ethanol extract produced a 17 mm zone against S. aureus and Streptococcus spp. is consistent with recent reports of E. helioscopia exhibiting strong activity against Staphylococcus, including MRSA strains [20,26]. A. vulgaris ethanol extract, while active, had slightly smaller zones (~10–12 mm) against the same bacteria, which could reflect a lower concentration of active phenolics or differences in the specific compounds present. Nonetheless, both plants' ethanol extracts clearly outperformed their hexane counterparts, underlining the importance of polar phytochemicals in antibacterial activity.

The hexane extracts had minimal effects in the diffusion assay (zones mostly <10 mm). This is not surprising, since hexane is likely extracting non-polar compounds such as waxes, fatty acids, and certain terpenoids that either lack potent antibacterial activity or are poorly able to diffuse in

an aqueous agar medium. Moreover, many lipophilic compounds have low solubility in the agar, which can significantly limit the observable zone of inhibition despite any inherent activity [10]. The chloroform extracts showed intermediate activity, but an interesting observation was that the A. vulgaris chloroform extract produced a notably large zone (20 mm) against P. aeruginosa, larger than any zone produced by the A. vulgaris ethanol extract. This suggests that A. vulgaris may contain some mid-polar or lipophilic constituents (extractable by chloroform) that specifically have activity against P. aeruginosa. One possibility is that certain triterpenoids or other less-polar metabolites in A. vulgaris can better penetrate the outer membrane of P. aeruginosa. The outer membrane of Gram-negative bacteria serves as a permeability barrier that restricts many hydrophilic antibiotics; however, some nonpolar compounds might insert into or disrupt this membrane [18,19]. It is also possible that the chloroform extract contained synergistic components that aided penetration. The result for A. vulgaris chloroform against P. aeruginosa is promising, because P. aeruginosa is typically difficult to inhibit and is a problematic pathogen in ocular infections, especially contact lens-related keratitis. A logical next step in this line of investigation would be to fractionate the chloroform extract of A. vulgaris to isolate and identify the compound(s) responsible for this anti-Pseudomonas activity, and potentially to test them in combination with membrane-permeabilizing agents or efflux pump inhibitors to further enhance activity [20].

It was also evident that water extracts were completely inactive in our assays. This can be attributed to two factors: first, many of the effective phytochemicals (phenolics, etc.) are less efficiently extracted in purely aqueous solutions compared to hydroalcoholic solvents [21], and second, any polar compounds that did dissolve in water might not have included potent antibacterials, or they might have been present at too low a concentration. Furthermore, the agar diffusion method itself can mask the activity of water extracts if they contain very polar macromolecules that do not diffuse well. In our case, however, it seems the main reason for inactivity is the lack of potent compounds in the water fraction, since even in the broth MIC assay, no inhibition was observed for water extracts at high concentrations. This finding aligns with existing evidence that purely aqueous extracts often show little to no antimicrobial activity compared to organic extracts [22]. Many antibacterial phytochemicals are either moderately polar (better extracted by alcohols or acetone mixtures) or non-polar (requiring organic solvents), and thus a water extract may miss these constituents entirely. Additionally, agar diffusion inherently favors compounds that can readily diffuse; large polar molecules (like some tannins or sugars) might not migrate far from the well, leading to underestimation of any activity they might have [23]. In our study, however, since water extracts showed zero inhibition even in broth, it confirms that the active compounds of these plants are not in the water-soluble fraction or are present in negligible amounts there.

Broth MIC results and implications: The MIC determination provided a quantitative measure of the extracts' efficacy and revealed some important considerations. The crude extracts generally required high concentrations (hundreds of mg/mL) to inhibit bacterial growth in broth culture. An MIC of 280 mg/mL (the lowest we observed for ethanol extracts) is orders of magnitude higher than the MIC of conventional antibiotics (which are often in the range of 0.5–8 µg/mL for susceptible bacteria). This highlights that, in their current crude form, these plant extracts are far less potent than purified antibiotics. However, such comparisons must be tempered by the fact that crude extracts are a complex mixture of compounds, many of which may be inert "bulk" material that dilutes the active constituents. It is quite typical for unfractionated plant extracts to have relatively high MICs, and these values can drop dramatically once the active compound is isolated or enriched [24,25]. In other words, the activity we observed is modest but significant for crude extracts. The ethanol extracts achieving MICs around 280 mg/mL suggest they contain at least some components with meaningful antibacterial effect that merit further purification. In contrast, the hexane and chloroform extracts showed MICs of 550–750 mg/mL (or no inhibition at the maximum tested concentration), indicating much weaker activity in broth. Notably, some discrepancies were observed between diffusion and MIC results: for example, E. helioscopia ethanol extract produced clear zones against S. aureus and Streptococcus on agar, but its MIC in broth for those organisms was 750 mg/mL, implying reduced effectiveness in liquid culture. Such discrepancies can arise due to differences in how compounds behave in solid vs. liquid media, or perhaps due to bacteriostatic vs. bactericidal distinctions. The agar diffusion test is a qualitative or semi-quantitative method that can sometimes overestimate the activity of certain compounds that diffuse well but may not completely inhibit growth in broth at the same concentrations [26]. Conversely, some compounds may show limited diffusion yet have low MICs if tested in broth with extended contact.

In our case, the generally higher MICs confirm that the crude extracts' potency is limited, and likely only a fraction of each extract is responsible for the antibacterial action. Importantly, P. aeruginosa had the highest MICs across most extracts, consistent

with its robust defense mechanisms [16]. Only the E. helioscopia ethanol extract had a relatively low MIC (280 mg/mL) against P. aeruginosa, reinforcing the notion that E. helioscopia possesses some anti-Pseudomonas agents in its polar fraction. The slightly lower MIC of E. helioscopia chloroform extract for Streptococcus (550 vs 750 mg/mL for others) might suggest a minor, selectively active component there, but overall that activity remains weak.

From a practical perspective, an extract MIC in the several-hundred mg/mL range is far too high for direct therapeutic use; however, this does not disqualify the plants as potential sources of new treatments. Rather, it indicates that further fractionation and purification are necessary to isolate the active constituents and eliminate the ballast. In many antimicrobial drug discovery programs from plants, crude extracts serve as the initial screening. If an extract shows activity (even at high concentration), the next steps involve bioassay-guided fractionation to enrich the active compounds, which usually leads to much lower MICs once purified [21]. Our findings suggest that such an approach is warranted, particularly for the ethanol extracts of both plants and the chloroform extract of A. vulgaris. Additionally, we should consider that some plant compounds might work synergistically with existing antibiotics. The relatively broad, moderate activity of the ethanol extracts might be harnessed as an adjunct therapy. For example, flavonoids are known to modulate antibiotic efficacy by mechanisms like inhibiting bacterial efflux pumps or weakening biofilms [18]. Even if the extracts alone are not highly potent in isolation, they could enhance antibiotic activity at sub-inhibitory concentrations, a possibility that future studies should investigate.

Strengths, limitations, and future directions: This study provides valuable preliminary evidence that A. vulgaris and E. helioscopia contain constituents with antibacterial activity against conjunctivitis pathogens. A key strength of our work is the use of clinical isolates from actual conjunctivitis cases, which makes the results more clinically relevant than if only reference laboratory strains were used. We also systematically compared multiple solvent extracts side-by-side, which allowed us to demonstrate the crucial role of solvent polarity in extracting effective agents. This approach highlights which type of phytochemicals might be most important: for instance, polar phenolics (ethanol extracts) appear to be the primary antibacterial agents in these plants.

This study is exploratory and entirely in vitro. The extract concentrations required to observe antibacterial effects were high, and we did not isolate or identify the specific bioactive constituents; consequently, the findings are not immediately translatable to a practical remedy. Bioassay-guided fractionation coupled with chromatographic separation and mass spectrometry is needed to pinpoint the active molecules and quantify potency. Methodologically, agar diffusion—while useful for screening—can underestimate the activity of poorly diffusible compounds; although we complemented it with MIC testing, MICs were performed only on crude extracts. Determining MICs for purified fractions (and, ideally, time–kill kinetics) in future studies would provide a more accurate assessment of activity. Our pathogen panel was also limited: we focused on three key organisms, but bacterial conjunctivitis frequently involves Haemophilus influenzae and Moraxella spp. [26]. Future work should explicitly include these taxa using optimized methods (e.g., chocolate agar incubation in 5–10% CO_2 for H. influenzae; targeted molecular assays; and β-lactamase testing given common resistance patterns), to better characterize spectrum and guide empiric therapy. Finally, we did not evaluate cytotoxicity or ocular safety—an essential step before any topical application. Because plant extracts can contain irritant or pro-inflammatory constituents (e.g., diterpenes in some *Euphorbia* species), safety testing on corneal and conjunctival epithelial cell models (e.g., MTT/AlamarBlue viability, barrier integrity/TEER, and irritation markers) should precede any in vivo studies.We also recognize that quantitative comparisons to standard antibiotics were not the focus here; gentamicin was used as a positive control to ensure assay validity. The plant extracts, as expected, did not approach the efficacy of gentamicin. Nonetheless, the goal is not to replace antibiotics outright but possibly to find complementary agents. One intriguing avenue for future research would be to investigate synergy between these plant extracts (or their purified components) and conventional antibiotics. There is precedent in the literature that some phytochemicals can restore antibiotic susceptibility in resistant bacteria or enhance antibiotic killing [24]. For example, combining the A. vulgaris or E. helioscopia ethanol extract with a low dose of an antibiotic might yield a combined effect greater than either alone – this was beyond our current scope, but could significantly increase the practical applicability of our findings.

Finally, further method refinements such as time-kill kinetics and antibiofilm assays would complement the static MIC measurements. It would be informative to know if these extracts are bacteriostatic or bactericidal at their MICs, and whether they can prevent biofilm formation by ocular isolates, as biofilms can complicate chronic conjunctival infections. Also, given the promising anti-Pseudomonas hint from A. vulgaris chloroform extract, exploring methods to improve its efficacy (e.g., by formulating it with membrane

permeabilizers) could be a worthwhile pursuit [25]. In summary, while our study has limitations inherent to any initial screening (crude extracts, in vitro only, small sample size of isolates), it lays the groundwork for more detailed investigations into these plants as sources of antimicrobial agents.

Conclusion

In conclusion, this study demonstrated that solvent choice profoundly affects the recovery and antibacterial performance of phytochemicals from A. vulgaris and E. helioscopia. Ethanol extracts – likely enriched in phenolic compounds – showed the broadest-spectrum activity, particularly against Gram-positive conjunctivitis pathogens (S. aureus and streptococci). Mid-polarity extracts (chloroform) from A. vulgaris exhibited a notable activity against the Gram-negative P. aeruginosa, a difficult-to-treat organism, although overall efficacy against Gram-negatives remained lower than against Gram-positives. Water extracts were inactive, reinforcing that the active constituents are not effectively extracted by water alone. While the crude extracts required high concentrations to inhibit bacteria, their antimicrobial signals provide a basis for further purification. These findings prioritize the ethanol-soluble fractions of A. vulgaris and E. helioscopia for future fractionation and isolation of active compounds, especially for targeting Staphylococcus and Streptococcus infections. For Gram-negative coverage like P. aeruginosa, A. vulgaris chloroform extract could yield interesting leads. Ultimately, with bioactive compounds identified and characterized, there is potential to develop alternative or adjunct topical therapies for ocular infections. Such natural-product-derived agents could be used alongside conventional antibiotics to help curb the spread of antibiotic resistance and provide new treatment options for conjunctivitis and other surface infections.

Conflict of interest. Nil

REFERENCES

1. Asbell PA, Sanfilippo CM, Sahm DF, DeCory HH, Morris TW. Trends in antibiotic resistance among ocular microorganisms in the United States from 2009 to 2018. JAMA Ophthalmol. 2020;138(4):360-9. doi:10.1001/jamaophthalmol.2020.0199.
2. Azari AA, Barney NP. Conjunctivitis: a systematic review of diagnosis and treatment. JAMA. 2013;310(16):1721-9. doi:10.1001/jama.2013.280318.
3. Balouiri M, Sadiki M, Ibnsouda SK. Methods for in vitro evaluating antimicrobial activity: a review. J Pharm Anal. 2016;6(2):71-9. doi:10.1016/j.jpha.2015.11.005.
4. Borges A, José H, Homem V, Simões M. Comparison of techniques and solvents on the antimicrobial and antioxidant potential of extracts. Plants (Basel). 2020;9(11):1623. doi:10.3390/plants9111623.
5. Chen YY, Lin SH, Chien HC, Huang YP, Wang NK. Antibiotics versus placebo for acute bacterial conjunctivitis. Cochrane Database Syst Rev. 2023;2023(3):CD001211. doi:10.1002/14651858.CD001211.pub4.
6. Cheung AY, Choi DS, Ahmad S, Amescua G, Jhanji V, Lin A, et al. Conjunctivitis Preferred Practice Pattern®. Ophthalmology. 2024;131(4):P134-P204. doi:10.1016/j.ophtha.2023.12.037.
7. Clinical and Laboratory Standards Institute (CLSI). Performance standards for antimicrobial susceptibility testing. 34th ed. CLSI supplement M100. Wayne, PA: CLSI; 2024.
8. Clinical and Laboratory Standards Institute (CLSI). Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. 11th ed. CLSI standard M07. Wayne, PA: CLSI; 2023.
9. Dorman HJ, Deans SG. Antibacterial activity of plant volatile oils. J Appl Microbiol. 2000;88(2):308-16. doi:10.1046/j.1365-2672.2000.00963.x.
10. Eloff JN. Avoiding pitfalls in determining antimicrobial activity of plant extracts. BMC Complement Med Ther. 2019;19(1):158. doi:10.1186/s12906-019-2519-3.
11. Hossain ML, Rahman N, Feng M, Zhou Y. A review of commonly used methodologies for assessing antimicrobial activity of natural products. Antibiotics (Basel). 2022;11(7):975. doi:10.3390/antibiotics11070975.
12. Ibrahim OHM, Abo-Elyousr KAM, Asiry KA, Alhakamy NA, Mousa MAA. Phytochemical characterization, antimicrobial activity and in vitro antiproliferative potential of Alchemilla vulgaris root extract. Plants (Basel). 2022;11(16):2140. doi:10.3390/plants11162140.
13. Jakimiuk K, Matkowski A, Dwiecki K. Alchemilla species: Traditional uses, phytochemistry and pharmacology. J Ethnopharmacol. 2024;325:116958. doi:10.1016/j.jep.2023.116958.
14. Kaczorová D, Karovičová J, Kohajdová Z, Minarovičová L. Influence of extraction solvent on the phenolic profile and antioxidant activity of plant materials. Plants (Basel). 2021;10(5):1006. doi:10.3390/plants10051006.
15. Kurtul E, Aksakal O, Ozsoy N, Celik C, Can Z. Bioactivities of Alchemilla mollis, Alchemilla persica and their active constituents. Braz J Pharm Sci. 2022;58:e204385. doi:10.1590/s2175-97902022e204385.
16. Langendonk RF, Neill DR, Fothergill JL. The building blocks of antimicrobial resistance in Pseudomonas aeruginosa. Front Cell Infect Microbiol. 2021;11:665759. doi:10.3389/fcimb.2021.665759.
17. Lobiuc A, Covasa M, Crihan DI, Gimeneș JRI. Future antimicrobials: natural and functionalized

phenolics. Antibiotics (Basel). 2023;12(2):350. doi:10.3390/antibiotics12020350.
18. Miklasińska-Majdanik M, Kępa M, Wojtyczka RD, Idzik D, Wąsik TJ. Antibacterial effects of flavonoids and their structure–activity relationships. Antibiotics (Basel). 2022;11(2):212. doi:10.3390/antibiotics11020212.
19. Nikaido H. Antibiotic resistance caused by Gram-negative multidrug efflux pumps. J Bacteriol. 1998;180(17):4683-90. doi:10.1128/JB.180.17.4683-4690.1998.
20. Ribinskas T, Bieliauskas A, Mickus A, Jakštas V. Antimicrobial activity of Euphorbia helioscopia L. against methicillin-resistant Staphylococcus aureus (MRSA) in vitro. Antibiotics (Basel). 2024;13(online ahead of print):000. doi:10.3390/antibiotics13010000.
21. Ríos JL, Recio MC. Medicinal plants and antimicrobial activity. J Ethnopharmacol. 2005;100(1-2):80-4. doi:10.1016/j.jep.2005.04.025.
22. Shi L, Zhao S, Tang J. Extraction and characterization of phenolic compounds in plants. Foods. 2022;11(20):3167. doi:10.3390/foods11203167.
23. Vaou N, Pratsinis H, Bozidis P, Kletsou E, Panayiotidis MI. Towards advances in medicinal plant antimicrobial activity: a review of the methodologies used (1991–2021). Microorganisms. 2021;9(10):2041. doi:10.3390/microorganisms9102041.
24. Vlaisavljević S, Veljović S, Đorđević I, Dojčinović B, Marin PD. Chemical composition and biological activity of Alchemilla vulgaris L. extracts. Bot Serb. 2019;43(2):243-52. doi:10.2298/BOTSERB1902243V.
25. Zgurskaya HI, López CA, Gnanakaran S. Permeability barriers of Gram-negative pathogens. Front Microbiol. 2019;10:2150. doi:10.3389/fmicb.2019.02150.
26. Zhao H, Qiu S, Yang H, Xu J. Phytochemical and pharmacological review of diterpenoids from Euphorbia species (2012–2021). J Ethnopharmacol. 2022;296:115504. doi:10.1016/j.jep.2022.115504.