



In Vitro Evaluation of Medicinal Plant Extracts Against *Corynebacterium pseudotuberculosis*: A Potential Remedy for Caseous Lymphadenitis in Small Ruminants

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ABSTRACT

Caseous lymphadenitis (CLA), caused by *Corynebacterium pseudotuberculosis*, is a chronic infectious disease of sheep and goats that causes substantial economic losses in Pakistan's livestock sector, which contributes 63.60% to agriculture and 14.97% to the national GDP. This study evaluated the antibacterial potential of three medicinal plants *Azadirachta indica* (Neem), *Moringa oleifera* (Sohanjana), and *Citrullus colocynthis* (Kortumma) against *C. pseudotuberculosis*. Leaves, bark, seeds, and fruit pulp were extracted using ethanol, distilled water, and acetone. Antibacterial activity was determined by well and disc diffusion methods on Mueller Hinton agar, following standard antibiotic protocols. Ethanol extracts exhibited the highest antibacterial activity, with *A. indica* showing the strongest inhibition, followed by *M. oleifera* and *C. colocynthis*. Statistical analysis (SPSS version 26.0) confirmed significant differences among treatments ($p < 0.05$). The results suggest that these plants contain potent bioactive compounds with promising antimicrobial efficacy. Overall, the findings support the potential of plant-derived remedies as natural and sustainable alternatives to conventional antibiotics for controlling *C. pseudotuberculosis* infections in livestock.

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INTRODUCTION

Pakistan's livestock industry is an important part of the country's national GDP and agriculture (Rehman et al. 2017). Sheep and goats are important animals in the livestock industry because they contribute a range of products, such as wool, milk, and meat (Mazinani et al. 2020). Pakistan stands as the fifth nation in terms of considerable milk production worldwide. Sheep and goats also contribute to milk production (Mazinani et al. 2020). Livestock has boosted the contribution to enhance the productivity of agriculture which is estimated to be 62.68% and 14.3 GDP for the year 2023 (Islam et al. 2023). Sheep and goat is a major activity in rural parts of Pakistan and about 8 million people are involved in livestock production, earning 40% of their income from the livestock sector (Afridi et al. 2009). Most of these animals is low in cholesterol and full of vitamin. But the disease prevalent in these animals resulting in heavy loss in Pakistan, Caseous Lymphadenitis (CLA) (Odhah et al. 2022).

C. pseudotuberculosis is gram-positive and is found in pleomorphic morphology (de Oliveira Zamprogna et al. 2021). The facultative pathogen ranges from 1.0 to 3.1 μm in length and 0.5 to 0.7 μm in width with either a coccoid or filamentous shape *C. pseudotuberculosis* can grow optimally at 37°C and pH 7.0 (Ahmed 2015). This disease is leading to high economic losses in all sectors that deal with the production of goats and sheep. CLA disrupts animal skin, meat, and wool production, thus forming abscesses (Mustaffa et al. 2025). Due to compromised physical condition or reduced fertility, sheep and goats become less marketable and lead to significant economic loss (Baird and Fontaine 2007).

CLA usually leads to the development of abscesses within lymph nodes. Their rigid wall structure leads to abscesses exhibiting antibiotic resistance (Fontaine and Baird 2008). Lipids present within the cell membrane and exotoxins facilitating the growth of abscesses characterize animal cells. While lipids provide bacteria with resistance to degradation, cellular enzymes are still not able to kill

them (Wright et al. 2005). In contrast, exotoxin facilitates bacteria to develop rapidly (Kobayashi et al. 2015). The abscesses enlarge until they burst, spilling the contents on the ground. The contents are made up of pus material with a yellow or greenish-white color. The pus material has germs that are capable of production of *C. pseudotuberculosis*, which is dangerous to other animals (Dopud et al. 2025).

Abscesses cannot be treated with antibiotics because their thick walls prevent the entry of antibiotic molecules (Wagner et al. 2006). Bacteria have developed resistance mechanisms against antibiotics due to their misuse and overuse. Bacteria have developed a variety of strategies to resist the action of antibiotics (Kuehl et al. 2020). There are approximately 78% of individuals across the globe that use traditional drugs to fight microorganisms. The plants and their extracts make up these drugs (Gurib-Fakim 2006).

Neem, or *Azadirachta indica*, is a tropical evergreen native to the Indian subcontinent, which includes Bangladesh, Nepal, India, and Pakistan (Kadir et al. 2024). It belongs to the Mahogany family (Meliaceae). Leaves, bark, and seeds of neem contain antiseptic, antiviral, anti-inflammatory, and antifungal bioactive compounds (Singh et al. 2021). Besides being a natural insecticide and source of agrochemical, neem has been extensively used in traditional medicine in the treatment of numerous diseases.

Antibacterial activity was tested by the well diffusion method and disc diffusion method (Zaidan et al. 2005). Glycerol-prepared bacterial stock cultures were used for inoculation. Bacterial cultures were inoculated carefully on Muller Hinton agar plates (Omokhua et al. 2018). Wells were made for the application of the well diffusion method, and 10 μ l of plant extracts consisting of fruit pulp, seed, and root extracts in different concentrations were loaded. To prepare the disc diffusion technique, the discs containing infusions of extracts, positive, and negative controls were inoculated onto the culture plates. Subsequently, all plates were incubated at 37 °C for 24 hours. Upon completion of the incubation period, the diameter of the inhibition zones was obtained (Mani et al. 2022).

MATERIALS AND METHODS

This experimental study was designed to evaluate the *in vitro* antibacterial activity of selected medicinal plants including *Azadirachta indica* (Neem), *Moringa oleifera* (Sohanjana), and *Citrullus colocynthis* (Kortumma)

against *Corynebacterium pseudotuberculosis*. The study was conducted in the Department of Microbiology, Faculty of Veterinary Sciences, Cholistan University of Veterinary and Animal Sciences (CUVAS), Bahawalpur. All procedures were performed under aseptic conditions, and each assay was conducted in triplicate for statistical reliability.

Collection and Identification of Medicinal Plants

Fresh leaves, bark, seeds and fruits of *A. indica*, *M. oleifera*, and *C. colocynthis* were collected from the experimental fields of CUVAS, Bahawalpur. All plant materials were washed with distilled water to remove dust and debris, shade-dried at room temperature ($25 \pm 2^\circ\text{C}$) for five days, and then grind into fine powder using a sterilized mortar and pestle (Oulaï et al. 2016). The powdered materials were stored in airtight containers at 4°C until extraction (Tashi et al. 2016).

Preparation of Plant Extracts

Plant extracts were prepared following the infusion method described by Gonçalves et al. (2013) with slight modifications. For each plant part, 2 g of powdered material was dissolved in 20 mL of three solvents including ethanol, acetone, and distilled water to obtain ethanolic, acetonetic, and aqueous extracts, respectively. The mixtures were agitated periodically and allowed to stand for 72 hours at room temperature shown in (Fig 1). Extracts were filtered using Whatman No. 1 filter paper, and the filtrates were concentrated under reduced pressure using a rotary evaporator at 40°C (van der Watt et al. 2001).

The concentrated extracts were stored in sterile glass vials at 4°C until further testing (Srivastava et al. 2007). A pure culture of *C. pseudotuberculosis* was obtained from the Department of Microbiology, CUVAS. The culture was revived in nutrient broth and then in Nutrient Agar, incubated at 37°C for 24 hours. Identification of the bacterium was confirmed through Gram staining, biochemical characterization, and colony morphology on blood agar (Dimri et al. 2020). The bacterial suspension was standardized to 0.5 McFarland turbidity standard (approximately 1.5×10^8 CFU/mL) before use in antimicrobial assays.

Antibacterial Assay

The antibacterial activity of the plant extracts was determined using both the agar well diffusion and disc diffusion methods as described by Mani et al. (2022), with minor modifications.



Fig 1: Collection, grinding, and extraction of *Azadirachta indica*, *Moringa oleifera*, and *Citrullus colocynthis* plant samples for antibacterial analysis against *Corynebacterium pseudotuberculosis*.



Fig 2: Determination of minimum inhibitory concentration (MIC) of plant extracts against *C. pseudotuberculosis* using the broth microdilution method in a 96-well microtiter plate.

Agar Well Diffusion Method

Mueller Hinton Agar (MHA) plates were inoculated uniformly with *C. pseudotuberculosis* using sterile cotton swabs. Wells of 6 mm diameter were punched aseptically into the agar using a sterile cork borer (Oladipo et al. 2015). A 100 μ L aliquot of each plant extract was added to the respective wells (prepared at concentrations of 25, 50, and 100 mg/mL). Distilled water served as the negative control, while gentamicin (10 μ g/mL) served as the positive control. Plates were incubated at 37°C for 24 hours, and zones of inhibition (in mm) were measured using a digital Vernier caliper.

Disc Diffusion Method

Sterile filter paper discs (6 mm diameter) were impregnated with 10 μ L of plant extract solutions and placed on MHA plates previously inoculated with the bacterial suspension (Othman et al. 2011). Control discs soaked with solvent (negative control) and antibiotic (positive control) were placed. After incubation at 37°C for 24 hours, the inhibition zones were measured, and mean values were recorded.

Microdilution Assay

The MIC of each extract was determined using the broth microdilution method (Liu et al. 2007). Serial dilutions (ranging from 12.5 to 200 mg/mL) of each extract were prepared in nutrient broth. Equal volumes of standardized bacterial suspension were added to each well of a 96-well microtiter plate as shown in Fig 2. After incubation at 37°C for 24 hours, turbidity was visually inspected. The lowest concentration showing no visible bacterial growth. To confirm bacterial inhibition, 10 μ L from each well was subcultured on MHA plates.

Antibiotic Susceptibility Testing

Antibiotic susceptibility of *C. pseudotuberculosis* was assessed using the Kirby Bauer disc diffusion method according to Clinical and Laboratory Standards Institute (CLSI, 2015) guidelines (Abebe et al. 2015). Antibiotic discs including oxacillin (1 μ g/mL), amoxicillin (25 μ g/mL) and gentamicin (10 μ g/mL) were placed on plates as shown in (Fig 3.). Plates were incubated at 37°C for 24 hours, and zones of inhibition were measured to determine resistance patterns.



Fig 3: Antibiotic susceptibility testing of *C. pseudotuberculosis* using the Kirby-Bauer disc diffusion method with oxacillin, amoxicillin, and gentamicin discs on Mueller–Hinton agar.

Statistical Analysis

Statistical analyses were conducted using one-way ANOVA in SPSS (version 26.0), with significance determined at $P < 0.05$ to assess differences among treatment means.

RESULTS

The study revealed the presence of *C. pseudotuberculosis*, the causative agent of caseous lymphadenitis (CLA) in sheep and goats across peri-urban areas of District Bahawalpur. The disease caused notable economic losses to livestock farmers. Microscopic examination confirmed the characteristic morphology of *C. pseudotuberculosis* (Fig 4).

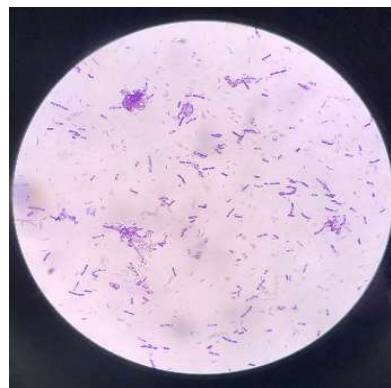


Fig 4: Microscopic examination of *C. pseudotuberculosis* morphology

Antibacterial Activity of *A. indica* and *M. oleifera*

Both the type of plant part ($F(5, 36) = 202.54, p < 0.05$) and solvent ($F(2, 36) = 518.09, p < 0.05$) significantly influenced antibacterial response. The interaction between the two factors was also significant ($F(10, 36) = 268.90, p < 0.05$), confirming that solvent choice altered plant-part efficacy.

As shown in Table 1, distilled water extracts exhibited the highest ZOI values compared to ethanol and acetone. *A. indica* leaves extracted in distilled water showed the strongest antibacterial effect (20 ± 0.9 mm), followed by *M. oleifera* leaves (18 ± 0.4 mm) and seeds (17 ± 0.8 mm). Ethanol and acetone extracts of *A. indica* seeds and

M. oleifera leaves showed minimal or no inhibition. Comparatively, standard antibiotics, Oxacillin (10 ± 0.8 mm) and Gentamicin (15 ± 0.5 mm) were less effective than several plant extracts, highlighting the strong antimicrobial potential of aqueous extracts of *A. indica* and *M. oleifera*.

Table 1: Comparison of zone of inhibition (ZOI mm) of *A. indica* (leaves, seeds and bark) and *M. oleifera* (leaves, seeds and bark) along with different solvents against *C. pseudotuberculosis*

Parts of plants	Solvents	Zone of inhibition (mm)
<i>A. indica</i> leaves	Ethanol	10 ± 0.4^{fg}
<i>A. indica</i> leaves	Acetone	08 ± 0.2^{hi}
<i>A. indica</i> leaves	Distilled Water	20 ± 0.9^a
<i>A. indica</i> seeds	Ethanol	0 ± 0^k
<i>A. indica</i> seeds	Acetone	07 ± 0.5^{ij}
<i>A. indica</i> seeds	Distilled Water	13 ± 0.7^d
<i>A. indica</i> bark	Ethanol	10 ± 0.6^{fg}
<i>A. indica</i> bark	Acetone	13 ± 0.8^d
<i>A. indica</i> bark	Distilled Water	07 ± 0.3^{ij}
<i>M.oleifera</i> leaves	Ethanol	12 ± 0.6^{de}
<i>M.oleifera</i> leaves	Acetone	0 ± 0^k
<i>M.oleifera</i> leaves	Distilled Water	18 ± 0.4^b
<i>M.oleifera</i> seeds	Ethanol	15 ± 0.9^c
<i>M.oleifera</i> seeds	Acetone	11 ± 0.7^{ef}
<i>M.oleifera</i> seeds	Distilled Water	17 ± 0.8^b
<i>M.oleifera</i> bark	Ethanol	06 ± 0.2^j
<i>M.oleifera</i> bark	Acetone	13 ± 0.5^d
<i>M.oleifera</i> bark	Distilled Water	07 ± 0.4^{sh}
Oxacillin	-	10 ± 0.8
Gentamicin	-	15 ± 0.5

Antibacterial Assay Confirmation

The disc diffusion assay further confirmed that aqueous extracts of *A. indica* and *M. oleifera* exhibited the most potent antibacterial activity, particularly in leaf and seed extracts (Sandhu et al. 2014). Solvent-dependent variations were observed, as some ethanol and acetone extracts displayed negligible inhibition zones (Fig 5).



Fig 5: Anti-bacterial tests of *A. indica*, *M. oleifera*, and antibiotics showing less or no zones of inhibition.

Minimum Inhibitory Concentration (MIC) of *A. indica* and *M. oleifera*

All factors individually and in combination, affected antibacterial activity. The best MIC values were observed in ethanolic and acetic extracts of bark ($5 \mu\text{L}$) for both *A. indica* and *M. oleifera*. Distilled water extracts required higher concentrations ($10\text{--}11 \mu\text{L}$) to achieve similar inhibition.

Overall, *M. oleifera* displayed slightly stronger broad-spectrum antibacterial activity than *A. indica*, and ethanol proved to be the most efficient solvent for extracting

antimicrobial agents. Bark extracts showed the greatest potency in both species.

Antibacterial Activity and Minimum Inhibitory Concentration (MIC) of *C. colocythis*

The MIC results demonstrated that plant parts alone did not show a statistically significant effect ($p = 0.060$), both solvent type ($p < 0.05$) and the interaction between plant part and solvent ($p < 0.05$) significantly influenced antibacterial activity. Solvent selection played a major role in bioactive compound extraction.

The highest ZOI (15 ± 0.9 mm) was observed in *C. colocythis* fruit extracted with acetone, comparable to Gentamicin (15 ± 0.5 mm). Seeds extracted with ethanol showed no activity (0 ± 0 mm) (Handal et al. 2000). Overall, the combination of fruit and acetone produced the strongest antibacterial response, followed by leaves extracted with ethanol. These findings confirm solvent and plant-part interactions as critical determinants of efficacy (Fig 5).

MIC values varied across parts and solvents, with seeds showing the strongest antibacterial activity at lower concentrations. The most notable MIC values were:

- Seeds + Ethanol ($7 \mu\text{L}$): MIC = $0.02640 \mu\text{L}$
- Seeds + Distilled Water ($2 \mu\text{L}$): MIC = $0.01460 \mu\text{L}$
- Fruit + Distilled Water ($5 \mu\text{L}$): MIC = $0.02620 \mu\text{L}$

These results highlight *C. colocythis* seeds as the most effective plant part, especially when extracted in ethanol or distilled water. Ethanol was the most reliable solvent overall, yielding the lowest MICs across plant parts.

Oxacillin showed optimal activity at $2 \mu\text{L}$ (MIC = $0.0660 \mu\text{L}$) and $5 \mu\text{L}$ (MIC = $0.0770 \mu\text{L}$), while Gentamicin showed the lowest MICs at $1\text{--}2 \mu\text{L}$ ($0.0230\text{--}0.0236 \mu\text{L}$). However, both displayed decreased efficacy at higher concentrations, possibly due to concentration-dependent resistance mechanisms.

Comparative Efficacy of Plant Extracts and Antibiotics

As shown in Table 2, therapeutic trials indicated that both antibiotics and plant extracts improved recovery outcomes compared to the untreated group.

These findings demonstrate that while antibiotics ensured the fastest recovery, medicinal plant extracts also exhibited strong therapeutic potential against *C. pseudotuberculosis*, reducing clinical symptoms, inflammation, and mortality.

Antibiotics often fail to reduce abscess formation in caseous lymphadenitis (CLA) in sheep and goats because their thick-walled abscesses prevent drug penetration. Therefore, herbal plants were investigated as alternative agents against *Corynebacterium pseudotuberculosis*, the causative organism of CLA (Odhah et al. 2022). Antibacterial activity was assessed using the disc diffusion assay. Among all extracts tested, *A. indica* leaves extracted with distilled water exhibited the highest antibacterial activity (ZOI = 20 ± 0.9 mm), followed by *M. oleifera* leaves with ethanol (18 ± 0.4 mm) and *C. colocythis* fruit with acetone (15 ± 0.7 mm). Extracts of *A. indica* seeds with ethanol, *M. oleifera* leaves with acetone, and *C. colocythis* seeds with ethanol showed no activity. Among controls, Oxacillin (15 ± 0.8 mm) and Gentamicin (10 ± 0.5 mm) showed effective inhibition. *A.*

Table 2: Comparative Efficacy of Antibiotics and Plant Extracts in Treating Infections

Groups	Treatment Description	Initial Rectal Temp (°C)	Final Rectal Temp (°C)	Abscess Size Reduction (%)	Survival Rate (%)	Clinical Observation Summary
G1	Control (No infection, no treatment)	38.5 ± 0.2	38.4 ± 0.1	N/A	100%	Normal, no signs of infection
G2	Infected, no treatment	39.7 ± 0.3	40.1 ± 0.4	0%	40%	Severe abscess, weight loss, lethargy
G3	Infected + Antibiotics (Gentamicin and Amoxicillin)	39.6 ± 0.2	38.7 ± 0.2	85%	100%	Recovery within 10-15 days, mild inflammation
G4	Infected + Medicinal Plants Extracts (<i>A. indica</i> , <i>M. oleifera</i> and <i>C. colocynthis</i>)	39.8 ± 0.4	38.9 ± 0.3	75%	100%	Moderate recovery, reduced swelling and fever

indica bark extracts in ethanol and acetone showed the lowest MIC (5 µL), while distilled water extracts were least effective (MIC = 10–11 µL). *M. oleifera* extracts displayed broader antibacterial activity, with the lowest MICs (5 µL) in ethanol and acetone, and even distilled water extracts performed.

DISCUSSION

A. indica. *C. colocynthis* seeds exhibited strong antibacterial potential at lower concentrations (as low as 0.0264 µL with ethanol and 0.0146 µL with water). Both antibiotics showed concentration-dependent effectiveness, decreasing at higher doses, emphasizing the importance of dose optimization to avoid resistance and maintain therapeutic efficacy.

Previous studies support these findings. Antibacterial activity of *A. indica* has been reported in the United Kingdom against *Staphylococcus aureus*, *Escherichia coli*, and *Bacillus subtilis* using ethanolic and acetic extracts (Alshahrani et al. 2022). Uma Devi and Lawrence (2014) recorded inhibition zones of 9 mm and 15 mm for *E. coli* and *Corynebacterium subtilis* respectively, while Gayathri, Bhakat, and Mohanty (2023) reported zones of 9 mm, 12 mm, and 20 mm for *M. oleifera* ethanolic, acetic, and aqueous extracts, respectively. Similarly, a study conducted in Faisalabad, Pakistan, observed inhibition zones of 5.0 mm, 7.1 mm, and 16.2 mm against *B. macerans*, *Azotobacter*, and *Corynebacterium* using ethanolic *C. colocynthis* extracts (Mazher et al. 2023).

MIC values obtained in this study are consistent with earlier findings. Agampodi et al. (2022) reported a MIC of 0.1 mg/mL for *A. indica* ethanolic extract against *E. coli* and *S. aureus*, which partially inhibited *P. aeruginosa* and *K. pneumoniae*. Similarly, *M. oleifera* extracts at 0.1 mg/mL inhibited *E. coli* and *S. aureus* but not *K. pneumoniae* or *P. aeruginosa*. *C. colocynthis* ethanolic extract showed inhibition at 0.15 mg/mL for *K. pneumoniae* and *P. aeruginosa*, while complete inhibition occurred at 0.25 mg/mL for all tested bacteria.

In a related study conducted in Nigeria, *A. indica* ethanolic leaf extracts showed antibacterial activity against *E. coli* and *S. aureus* with inhibition zones of 14.4 mm and 8.5 mm, respectively (Nigussie et al. 2021). The difference in inhibition zones between disc diffusion (14.4 mm) and paper diffusion (9 mm) methods was attributed to greater extract diffusion in agar using the disc diffusion technique. Methanolic extracts of *M. oleifera* leaves demonstrated high activity against *E. coli* (21.5 mm) but none against *P.*

aeruginosa and *S. pneumoniae* (Abalaka et al. 2022). However, methanolic latex extracts showed inhibition of 18.5 mm for *P. aeruginosa* and *S. pneumoniae*. Strong antibacterial effects were also observed against Gram-positive bacteria such as *B. cereus*, *S. epidermidis*, and *B. subtilis*, with inhibition zones of 19.5 mm, 23.5 mm, and 22.0 mm, respectively.

No antibacterial activity was reported for *C. colocynthis* methanolic leaf extracts against Gram-positive bacteria, but inhibition zones appeared against Gram-negative bacteria, including *E. coli*. MIC values for methanolic leaf extracts were 0.75 mg/mL for *S. epidermidis*, 0.50 mg/mL for *B. cereus*, and 0.25 mg/mL for *E. coli*. Various solvents such as methanol, chloroform, alcohol, and benzene have been reported to effectively extract phytochemicals from different plant parts like root bark, stem, and flowers, all possessing notable antibacterial potential (Mehmood et al. 2022). The absence of antibacterial activity in *A. indica* seed extracts and *M. oleifera* leaves (ethanol) observed in this study may be attributed to the absence of bioactive compounds or poor solvent extraction efficiency. Ethanol generally extracts curcumin and related phenolic compounds known for their antibacterial characteristics, yet its limited performance here suggests plant part–solvent specificity. Hence, the results demonstrate that both the choice of plant part and solvent significantly influence antibacterial potency against *C. pseudotuberculosis*, and ethanol remains the most effective solvent for extracting antibacterial phytochemicals.

Conclusion

This study demonstrated that *Azadirachta indica*, *Moringa oleifera*, and *Citrullus colocynthis* possess strong antibacterial activity against *Corynebacterium pseudotuberculosis*, the causative agent of caseous lymphadenitis in sheep and goats. Among the tested extracts, aqueous *A. indica* leaves, ethanolic *M. oleifera* leaves, and acetic *C. colocynthis* fruits showed the highest activity. Solvent type significantly influenced bioactive compound extraction and antibacterial efficacy. Statistical analysis confirmed that plant species, plant part, and solvent had significant effects ($p < 0.05$). Overall, *A. indica* and *M. oleifera* displayed broad-spectrum activity comparable to standard antibiotics, highlighting their potential as sustainable, natural alternatives for livestock disease management.

DECLARATION

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