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Comparative Evaluation of the Antibacterial Activity of *Curcuma longa* and *Calotropis procera* against *Corynebacterium pseudotuberculosis*

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Abstract: Livestock sector contributes 14% share in national GDP of the country. Population of sheep and goats in the country is 31.9 million and 82.5 million, respectively. Caseous lymphadenitis (CLA) is an important disease of sheep and goats that is posing huge economic losses to livestock farmers. *Corynebacterium pseudotuberculosis* is a gram-positive bacterium, acts as etiological agent of CLA which results in abscesses formation in different body parts. In the present study, antibacterial activity of ethanolic leave's extracts of *Calotropis procera* and *Curcuma longa* fresh rhizomes was checked against *C. pseudotuberculosis* that was isolated from pus samples collected from infected sheep (n=75) and goats (n=75) from different rural areas of district Bahawalpur. The zone of inhibition (ZOI) of extract of *Curcuma longa* was significantly higher (6.4mm ± 1.6, $P<0.05$) than ZOI of extract of *Calotropis procera*. Optical density (OD) values were measured at 0-hours, 6-hours and 24-hours at 550 nm wavelength. The MIC values measured at 24-hours for *Curcuma longa* and *Calotropis procera* were 0.0782 µg/ml and 0.3125 µg/ml, respectively and at 6-hours 0.156 µg/ml and 0.625 µg/ml were for *Curcuma longa* and *Calotropis procera*, respectively. The MIC values of extract of *Curcuma longa* showed more significant antibacterial effect (t-3.530, $P<0.05$) than the extract of *Calotropis procera* (t-1.893, $P>0.05$). The study recommends the use of ethanolic extracts of *Curcuma longa* to treat CLA in sheep and goats in rural areas and further herbal and pharmacological effect be studied to formulate a compact medicine.

Keywords: Herbal Plants; *Curcuma longa*; *Calotropis procera*; *C. pseudotuberculosis*; CLA

1. Introduction

Livestock plays a vital role in the economy of Pakistan, contributing approximately 14% to the national GDP (Pakistan Economic Survey 2023). Sheep and goats are vital livestock animals because they deliver essential products which include meat and milk and wool (1). The products provide essential protein and nutritional value, while wool serves as a primary material for textile manufacturing. Pakistan ranks among the leading countries in milk production globally, with small ruminants contributing substantially to this

sector. Rural communities rely on livestock farming as their main income source, which employs millions of people from all age groups including men and women and children (2). Sheep and goat farming is especially important because farmers need to spend less money to raise these animals, which produce marketable meat within a short time. The meat has nutritional advantages because it contains low cholesterol levels and high vitamin content, while the milk contains small fat globules which make it easy to digest. The main reason livestock productivity decreases is through infectious diseases, which result in lower animal growth and fertility and market value, which leads to financial losses (3).

The primary disease which impacts sheep and goats occurs through caseous lymphadenitis (CLA) which results from infection with *Corynebacterium pseudotuberculosis*. The disease produces abscesses which develop within lymph nodes and internal organs, causing skin damage and reduced wool quality and decreased meat production (4). Infected animals show physical weakness and lesser productivity, which results in worldwide economic damages. *C. pseudotuberculosis* features a Gram-positive bacterial structure which shows multiple forms between coccoid and filamentous shapes. The organism grows best at 37°C with a neutral pH value because it functions as a facultative pathogen (5). The organism has fimbriae which help with adhesion, but it lacks both flagella and a protective capsule. The organism can grow on brain heart infusion agar and blood agar media, while its growth process can be improved through the addition of yeast extract. The bacterium was first identified in the late 19th century and later classified under the genus *Corynebacterium* due to its morphological and biochemical similarities with related species (6).

The development of CLA begins with the establishment of thick-walled abscesses which stop antibiotics from entering the infection sites. The abscesses contain pus which has a high bacterial content that can spread through the environment when the abscesses break open, which aids in spreading the disease (7). Infection most frequently happens through skin abrasions, but contaminated feed and other routes also serve as infection pathways. The disease shows both external swelling of lymph nodes and internal body infections that affect different organs including the lungs, which cause symptoms such as chronic cough and weight loss and breathing difficulties. *C. pseudotuberculosis* identification requires the use of three different diagnostic methods which include microscopic examination and biochemical tests and molecular analysis (8). The Neisser and Albert staining methods provide a means to detect specific metachromatic granules through their staining properties, while bacterial cultures enable isolation of the bacteria. The polymerase chain reaction (PCR) molecular technique provides more precise results than traditional testing methods which use conventional approaches. The bacterium establishes its presence in host cells that include macrophages, which results in ongoing and repeat infections (9).

The virulence of C. pseudotuberculosis depends on its two main virulence factors which include phospholipase D and cytotoxic lipids. These components enable bacteria to survive through increased vascular permeability which allows them to infect multiple areas of the host body (10). The organism exists as a soil-borne pathogen because it can survive in the environment after abscesses release its infectious material. Insects function as mechanical vectors because they help spread diseases that affect different animal species. The treatment of CLA becomes difficult because antibiotics cannot effectively reach the bacteria inside abscesses due to their protective thick walls (11). Doctors typically use physical drainage to remove abscesses and then apply antiseptics while reserving antibiotics for secondary infection protection. The misuse and excessive use of antibiotics has created a situation where antimicrobial resistance becomes more difficult to control which leads to challenges in controlling diseases. Developing countries face a critical situation because people there have limited access to both regulated medical treatment and veterinary services (12). Researchers increasingly explore different treatment methods which include medicinal plants as their focus. Traditional medicine serves as the primary healthcare system for most of the global population whereas traditional medicine uses plant extracts that contain antimicrobial properties. People consider natural products to be safer options which cost less money and protect the environment when compared to synthetic antibiotics (13).

Turmeric (*Curcuma longa*) belongs to the *Zingiberaceae* family and people use it as both a culinary spice and a healing herb. The plant contains curcumin which acts as a bioactive compound that exhibits antiviral properties and antifungal effects and antimicrobial activity and anti-inflammatory effects and anticancer abilities (14). The antibacterial activity and antifungal activity of curcumin make turmeric a suitable option for use in infection control. Turmeric functions as an antimicrobial agent while people use it in traditional medicine to treat various skin infections and wound healing and other medical conditions (15). *Calotropis*

procera serves as an essential medicinal herb that belongs to the Asclepiadaceae family. The plant grows across tropical and subtropical regions while its milky latex demonstrates powerful antimicrobial capabilities. People have used extracts from its leaves and other parts to treat multiple medical conditions which include skin infections and respiratory disorders and abscesses. The plant has multiple bioactive compounds which include alkaloids and flavonoids and tannins and terpenoids that create its medicinal properties (16).

The need for alternative treatments has become essential because patients experience side effects from antibiotics while bacteria develop resistance to these drugs. The natural bioactive properties of *Curcuma longa* and *Calotropis procera* make these medicinal plants suitable as effective treatment options. The researchers aim to assess the antibacterial properties of plant extracts against pathogens that cause caseous lymphadenitis in order to create safer and more effective disease control methods (17).

2. Materials and Methods

2.1. Study area and Sample Collection

The present study was conducted in peri-urban and rural areas of district Bahawalpur, Pakistan. Livestock farming is widely practiced in this region which makes it an appropriate area for sampling. The researchers gathered a total of 150 samples which included 75 samples from sheep and 75 samples from goats. Pus samples were obtained from abscesses of animals that the veterinarians suspected to have lymphadenitis. The researchers collected approximately 1 mL of pus in an aseptic manner by using sterile disposable syringes. The researchers used ice-packed coolers to transport all collected samples to the Microbiology Laboratory.

2.2. Isolation and Identification of the Organism

The microbiology laboratory maintained sterile conditions to perform the isolation of *C. pseudotuberculosis*. The researchers first added samples to nutrient broth which they then incubated at 37°C for 24 hours to check for bacterial growth based on turbidity results. The researchers then transferred the cultures to nutrient agar plates which they incubated at 37°C for 24 hours. The researchers used blood agar media as a selective isolation method to sub-culture colonies which they wanted to purify (18). The researchers conducted incubation procedures to study colony development. The researchers used Gram staining methods to conduct preliminary identification. The oil immersion microscope examination at 100× revealed the presence of Gram-positive coccobacilli which confirmed the morphological traits of *C. pseudotuberculosis*. Biochemical tests which included the catalase test, urease test, indole tests were used for the further identification of *C. pseudotuberculosis*.

2.3. Herbal Collection and Preparation of Plant Extracts

Fresh leaves of *Calotropis procera* and *Curcuma longa* were collected from the field area of Cholistan University of Veterinary and Animal Sciences. The collected plant materials were thoroughly washed with clean distilled water to remove dust particles, soil debris, and other contaminants. After washing, the leaves were air-dried at room temperature under shaded conditions for several days until complete removal of moisture was achieved (Figure 1). Shade drying was preferred to preserve the bioactive phytochemical constituents that may be sensitive to direct sunlight and excessive heat.

The dried leaves were then finely crushed into small particles using a sterile mortar and pestle to increase the surface area for efficient solvent extraction. For the preparation of ethanolic extracts, 65 g of the powdered plant material from each plant species was weighed separately and mixed with 165 mL of ethanol in sterile glass containers. The extraction process was carried out using the maceration technique, in which the mixtures were stored at 4°C for 24 hours with intermittent shaking to facilitate maximum extraction of phytoconstituents (Figure 2).

Following maceration, the mixtures were filtered through sterile Whatman No. 1 filter paper to separate the plant residues from the solvent extracts. The obtained filtrates containing the crude ethanolic extracts were transferred into sterile airtight containers and preserved at 4°C until further use in antimicrobial assays.

For antimicrobial susceptibility testing, sterile filter paper discs were prepared using a punch machine to obtain discs measuring 5 mm in diameter. The discs were sterilized in an autoclave at 121°C under 15

psi pressure for 15 minutes to ensure complete elimination of microbial contaminants. After sterilization, the discs were aseptically stored in sterile containers until their application in antimicrobial activity testing.

Figure 1. (a) Collection of leaves of *Calotropis procera* from desert fields of the Cholistan region, District Bahawalpur. (b) Separation of leaves from stem branches to obtain clean leaf samples. (c) Grinding of leaves using a mortar and pestle to obtain a fine powder for extract preparation. (d) Collection of rhizomes of *Curcuma longa* from a local vegetable market for experimental use.

Figure 2. Extraction of leaves of *Calotropis procera* and rhizomes of *Curcuma longa* using the maceration method for preparation of crude plant extracts

2.4. Disc Diffusion Assay

The antibacterial activity of plant extracts was evaluated using the Kirby-Bauer disc diffusion method. The preparation of Mueller Hinton agar involved dissolving the required amount in distilled water which underwent sterilization through autoclaving at 121°C for 15 minutes (19). A *C. pseudotuberculosis* overnight culture was prepared by adjusting its concentration to 1.5×10^8 CFU/mL according to McFarland standards (20). The bacterial inoculum was spread evenly on Mueller Hinton agar plates through the use of sterile cotton swabs. The researchers applied sterilized filter paper discs with *Calotropis procera* and *Curcuma longa* extracts which were allowed to air dry afterward. The researchers used sterile forceps to position these discs on the inoculated agar surface. The researchers incubated the plates at 37°C for 24 hours after which they measured zones of inhibition to assess antibacterial activity.

2.5. Determination of MIC

The broth microdilution method established the Minimum Inhibitory Concentration (MIC) for plant extracts. The *Corynebacterium pseudotuberculosis* overnight culture was adjusted to 1.5×10^8 CFU/mL which they incubated until the bacteria reached complete growth. The researchers created a 96-well microtiter

plate by combining 50 µL nutrient broth with 50 µL bacterial inoculum and different plant extract concentrations in each well. The plate was incubated at 37°C for 24 hours. Optical density (OD) values were recorded using an ELISA reader for confirmation Figure 3.

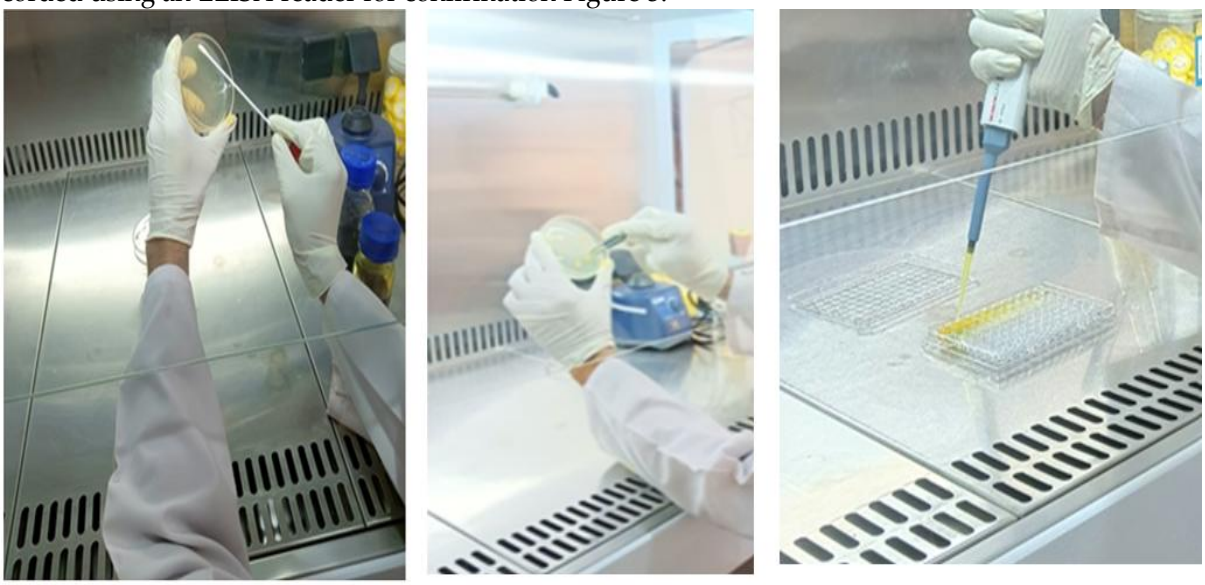

Figure 3. (a) Swabbing of *Corynebacterium pseudotuberculosis* culture onto solidified agar under a biosafety level-2 (BSL-2) cabinet using appropriate personal protective equipment (PPE). (b) Placement of sterile discs impregnated with plant extracts of *Calotropis procera* and *Curcuma longa* onto agar plates inoculated with *C. pseudotuberculosis*. (c) Dispensing of reagents into a 96-well microtiter plate for determination of minimum inhibitory concentration (MIC) of C. procera and C. longa extracts following standard microbiological protocols.

2.6. Statistical Analysis

All experiments were performed in triplicate, and results were expressed as mean ± standard deviation (SD). Significant differences among treatment groups were determined using one-way ANOVA at $p < 0.05$. Data was analyzed using SPSS (V. 26.0).

3. Results

3.1. Characterization, Morphological and Biochemical Confirmation of *C. pseudotuberculosis*

C. pseudotuberculosis was successfully isolated from 46 sheep and goat samples which made up the total of 150 samples that were collected. Bacterial growth developed when the nutrient broth showed its initial turbidity to the point of visible growth. The nutrient agar showed colonies that developed into small dry whitish forms, but blood agar subculturing produced distinctive colonies which researchers used to identify the organisms. The Gram staining procedure showed that the sample contained Gram-positive coccobacilli which researchers examined through microscopic observation as demonstrated in Figure 1. The organism was successfully isolated from the samples and its preliminary identification was confirmed by these findings.

The isolates showed positive results for the catalase test, which produced bubble formation, and the urease test, which produced bubble formation and pink color development. The indole test showed negative results because there was no formation of a red ring.

Molecular confirmation was carried out using modified 16S rRNA gene-specific primers: 16S-F (5′-ACCGCACTTTAGTGTGTGTG-3′) and 16S-R (5′-TCTCTACGCCGATCTTGTAT-3′). PCR amplification was performed in a thermal cycler (C1000 Touch™, Bio-Rad, USA) under optimized cycling conditions: initial denaturation at 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 1 min, annealing at 58 °C for 40 s, and extension at 68 °C for 90 s, with a final extension at 68 °C for 7 min. The amplified PCR products were confirmed by agarose gel electrophoresis using a Sub-Cell® GT system (Bio-Rad, USA) to verify the presence of the expected 16S rRNA gene fragment following protocol (21).

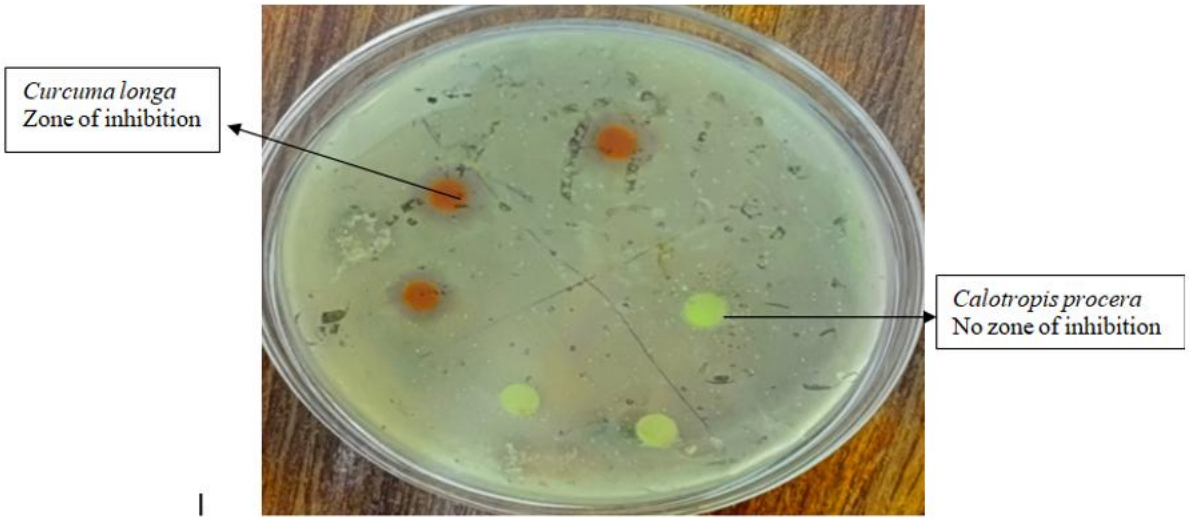

Figure 4. Zones of inhibition produced by extracts of *Calotropis procera* and *Curcuma longa* on solidified agar against the *C. pseudotuberculosis*. *Curcuma longa* extract exhibited a greater zone of inhibition compared to *Calotropis procera*, indicating higher antimicrobial activity.

3.2. Disc Diffusion Assay Results

The antibacterial activity of the ethanolic extracts of *Curcuma longa* and *Calotropis procera* was evaluated using the disc diffusion method against *Corynebacterium pseudotuberculosis* infection isolates. The results revealed that the ethanolic extract of *Curcuma longa* exhibited antibacterial activity by producing a measurable zone of inhibition with an average diameter of 5.4 mm around the impregnated disc, indicating its inhibitory effect against the tested bacterial isolate (Figure 4).

In contrast, the ethanolic extract of *Calotropis procera* did not produce any visible zone of inhibition around the discs during the incubation period, suggesting the absence of detectable antibacterial activity against the tested organism under the experimental conditions employed in this study. The observed variation in antibacterial efficacy between the two medicinal plant extracts may be attributed to differences in their phytochemical composition and bioactive constituents.

3.3. Minimum Inhibitory Concentration (MIC)

The MIC of the ethanolic extracts of *Curcuma longa* and *Calotropis procera* was determined using the broth microdilution method, as illustrated in Figures 5–7. Serial two-fold dilutions of each extract were prepared and inoculated with the test bacterial suspension under standardized conditions to evaluate concentration-dependent inhibition of bacterial growth.

The extract of *Curcuma longa* exhibited a strong dose-dependent antibacterial effect, with markedly lower MIC values observed over time. At 6 hours of incubation, the MIC was recorded at 0.156 μg/mL, while at 24 hours it further decreased to 0.0782 μg/mL, indicating enhanced inhibitory activity and sustained suppression of bacterial proliferation. These findings suggest that *Curcuma longa* possesses potent bioactive constituents capable of exerting prolonged bacteriostatic or bactericidal effects against the tested organism.

In comparison, the extract of *Calotropis procera* demonstrated comparatively weaker antibacterial activity, as reflected by higher MIC values. The MIC was determined to be 0.625 μg/mL at 6 hours and decreased to 0.3125 μg/mL at 24 hours. Although a reduction in MIC over time was observed, the inhibitory effect remained significantly lower than that of *Curcuma longa*, indicating reduced efficacy against the bacterial strain under investigation.

Overall, the comparative MIC analysis highlights that *Curcuma longa* exhibits superior antibacterial potential relative to *Calotropis procera*, suggesting a higher concentration of active phytochemicals responsible for antimicrobial activity.

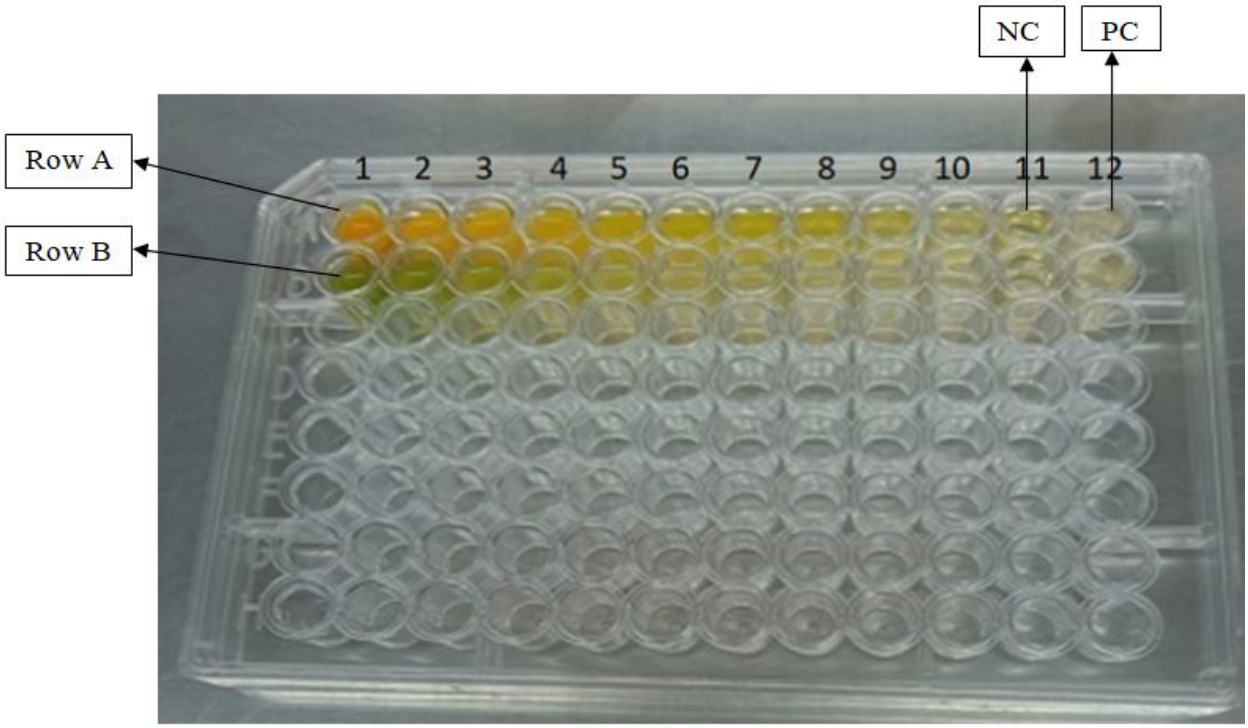

Figure 5. Observation of turbidity and color change in a 96-well microtiter plate after 24 hours of incubation for determination of antimicrobial activity. Wells A1–A10 contained extracts of *Curcuma longa* rhizomes, while wells B1–B10 contained extracts of *Calotropis procera* leaves. Wells A11 and B11 served as negative controls (NC), whereas wells A12 and B12 served as positive controls (PC).

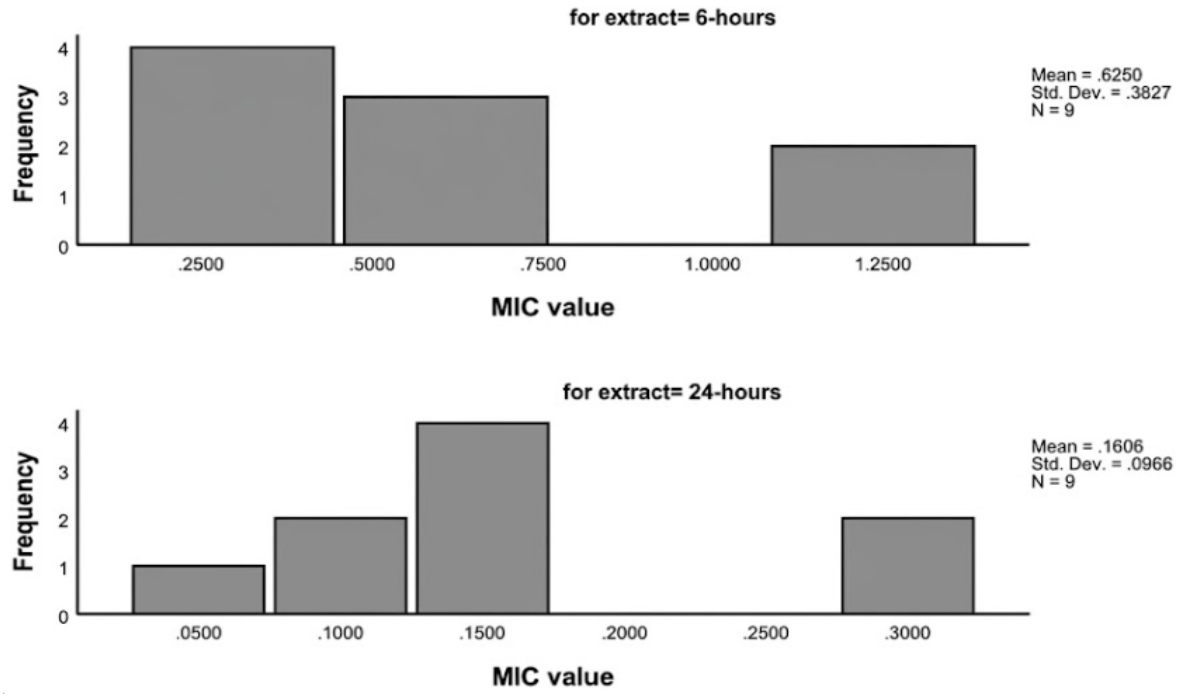

Figure 6. The plots illustrate the MIC frequency profile of *Curcuma longa* for 6-hour and 24-hour extraction periods (N=9). Values on the x-axis represent the concentration threshold for growth inhibition, with the 24-hour extract demonstrating a more concentrated distribution at lower MIC levels.

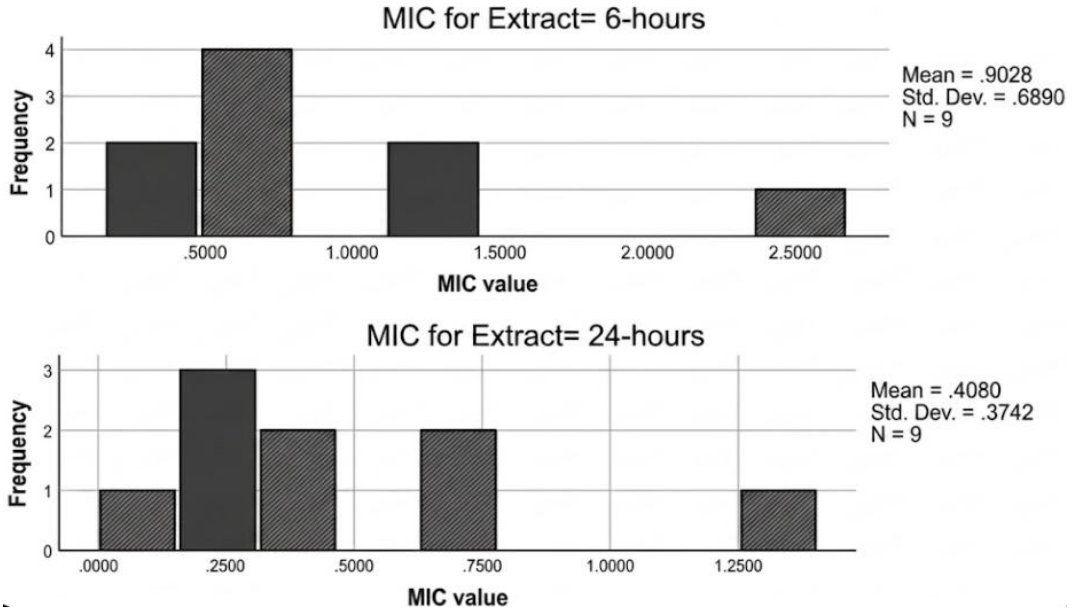

Figure 7. MIC value of *Calotropis procera* for 6-hour (top) and 24-hour (bottom) extract treatments. Data labels indicate the mean, standard deviation, and sample size (N=9) for each experimental condition.

Among the total samples analyzed, 17 out of 75 (22.7%) sheep samples and 29 out of 75 (38.7%) goat samples were found positive for *C. pseudotuberculosis*. The overall prevalence was higher in goats compared to sheep as shown in Figure 8.

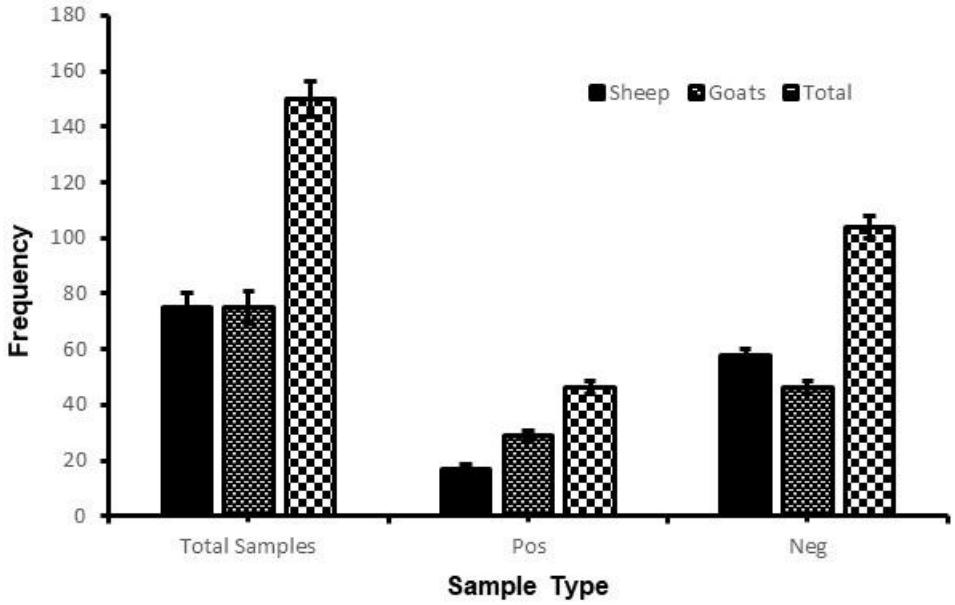

Figure 8. Distribution of collected samples from sheep and goats, including the overall sample frequency used for the isolation of *Corynebacterium pseudotuberculosis* infection.

Statistical analysis confirmed that the antibacterial effect of *Curcuma longa* was significant ($P < 0.05$), whereas *Calotropis procera* did not show a significant effect ($P > 0.05$).

4. Conclusions

The present study highlights the prevalence and successful isolation of *Corynebacterium pseudotuberculosis* from sheep and goats in the peri-urban areas of Bahawalpur, confirming its role as a significant pathogen responsible for caseous lymphadenitis. Goats show higher susceptibility to the disease because of their higher disease rates which require implementation of proper control methods for small ruminants. The antibacterial evaluation of plant extracts demonstrated that *Curcuma longa* possesses notable inhibitory activity ($P<0.05$) against *C. pseudotuberculosis* through disc diffusion and MIC testing.

The experimental conditions demonstrated that *Calotropis procera* did not produce any significant antibacterial activity (P>0.05). The findings demonstrate that turmeric functions as a natural antibacterial treatment because its bioactive compound curcumin possesses antibacterial properties. The study shows that plant-based antimicrobial agents together with *Curcuma longa* provide an effective solution to treat infections when standard antibiotics fail to work. The therapeutic effects of medicinal plants require verification through further research that should assess various extraction techniques and concentration levels together with in vivo testing.

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Data Availability Statement: The data supporting the findings of this study are available from the corresponding author upon reasonable request.

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Conflicts of Interest

The authors declare that there is no conflict of interest. This work did not get any external funding; therefore, no funding agency had a role in the whole plan of the study, the collection, the analysis or the interpretation of data, not even in the writing of the manuscript.

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