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COMPARATIVE EVALUATION OF ANTIMICROBIAL ACTIVITY AND STABILITY OF LYOPHILIZED COW URINE AND METHANOLIC COW DUNG EXTRACTS AGAINST CLINICALLY RELEVANT ATCC PATHOGENS

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ABSTRACT

The increasing prevalence of antimicrobial resistance (AMR) has intensified the search for sustainable and natural alternatives to conventional antibiotics. In the present study, we evaluated the antimicrobial potential and storage stability of lyophilized and methanolic cow dung extracts derived from organically raised Gir cows. Extracts were prepared under sterile and standardized conditions and assessed against four clinically significant ATCC reference strains: *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923, and *Salmonella typhimurium* ATCC 14028. Antimicrobial activity was determined using agar well diffusion and broth microdilution assays in accordance with the Clinical and Laboratory Standards Institute guidelines. Phytochemical analysis revealed the presence of phenolics, flavonoids, and alkaloids in both extracts, with higher phenolic content observed in the cow dung extract (78.9 ± 5.1 mg GAE/g) than in the cow urine extract (45.6 ± 3.2 mg GAE/g). Despite this, the cow urine extract demonstrated superior antimicrobial activity, producing larger zones of inhibition (12.2–16.0 mm) and lower minimum inhibitory concentration (MIC) values (0.25–0.50 mg/ml) than the cow dung extract (11.5–13.4 mm; 0.50–0.75 mg/ml). The greatest activity was observed against *E. coli*, with the cow urine extract showing a 50% lower MIC than the dung extract. Gentamicin controls confirmed the assay validity and exhibited substantially higher potency, as expected. Stability studies indicated that both extracts retained >90–95% of their initial antimicrobial activity after 12 weeks at -20 °C, with minimal loss (<10%). However, storage at room temperature (~25 °C) resulted in moderate activity reductions (15-25%) within four weeks, highlighting the importance of cold storage for bioactive preservation. Overall, our findings demonstrate that lyophilized cow urine extract exhibits stronger antimicrobial efficacy than methanolic cow dung extract against major gram-positive and gram-negative pathogens. These results support the potential application of bovine-derived bioactive extracts as eco-friendly antimicrobial agents and contribute to sustainable waste valorization strategies for combating antimicrobial resistance.

KEYWORDS: Cow urine extract, Cow dung extract, Antimicrobial activity, Agar well diffusion assay, Minimum inhibitory concentration (MIC)

INTRODUCTION

The rapid emergence of antimicrobial resistance (AMR) has become a global public health crisis, threatening the effectiveness of conventional antibiotics and increasing the burden of infectious

diseases worldwide. According to the World Health Organization (WHO), priority pathogens, such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Salmonella Typhimurium*, are increasingly associated with multidrug resistance, complicating treatment strategies in both clinical and agricultural settings (Matheou *et al*., 2025; Ogunniyi *et al*., 2017).

Traditional livestock-derived biomaterials, particularly cow urine and dung, have long been utilized in indigenous agricultural and medicinal practices (Gupta *et al*., 2016). Scientific investigations in recent years have begun to validate these traditional claims by identifying bioactive compounds, such as phenolics, flavonoids, alkaloids, antimicrobial peptides, and volatile metabolites, within bovine waste products (Pourreza, 2013; Sun and Shahrajabian, 2023). Studies have demonstrated that cow urine contains biologically active metabolites capable of exerting antibacterial, antioxidant, and Immunomodulatory effects. Similarly, cow dung, enriched with plant-derived secondary metabolites and microbial bioactive compounds, has shown potential antimicrobial and biocontrol properties.

Despite iṅcreasing interest, comparative evaluations of standardized lyophilized cow urine and methanolic cow dung extracts against clinically relevant reference strains remain limited. Furthermore, stability profiling under different storage conditions is critical for practical applications in pharmaceutical, agricultural, and bio-preservative contexts. The preservation of bioactivity during storage directly influences product feasibility, shelf life, and commercialization potential (Javadi *et al*., 2021). Therefore, the present study was designed to (i) prepare and standardize lyophilized cow urine and methanolic cow dung extracts from organically raised Gir breed cows, (ii) evaluate their antimicrobial activity against four clinically significant ATCC reference strains representing gram-positive and gram-negative pathogens, (iii) determine minimum inhibitory concentrations (MICs) following Clinical and Laboratory Standard Institute (CLSI) guidelines, and (iv) assess extract stability under frozen (–20 °C) and room temperature conditions over defined time intervals (Javadi *et al*., 2021; Kebede *et al*., 2021).

This experimental study was designed to comparatively evaluate the antimicrobial efficacy and storage stability of lyophilized and methanolic cow dung extracts obtained from organically maintained Gir cows. All experiments were performed under aseptic laboratory conditions in accordance with the Clinical and Laboratory Standards Institute guidelines for antimicrobial susceptibility testing (Balakrishnan and Kumar Subramanian, 2024; El- Hadary and Taha, 2020; Javed *et al*., 2022).

This study integrated phytochemical characterization, antimicrobial susceptibility testing, and stability assessment to comprehensively evaluate bovine-derived natural extracts as potential alternative antimicrobial agents. These findings contribute to sustainable waste valorization strategies and offer insights into the development of eco-friendly antimicrobial formulations aligned with global efforts to combat antimicrobial resistance.

MATERIALS AND METHODS

Sample Collection and Preparation

Urine sample

Fresh midstream urine samples were collected from five healthy Gir cows (aged 3–5 years) maintained at a certified organic farm in Thoriyali, Gujarat, India. The animals were exclusively fed certified organic feed and had not received antibiotic treatment for at least 90 days prior to sample collection to eliminate interference from antimicrobial residues.

Urine samples were collected during the morning milking sessions using sterile, autoclavable polypropylene containers. Samples were immediately transported to the laboratory under chilled conditions and subjected to sequential filtration through 0.45- and 0.22-µm sterile membrane filters (Millipore) to remove particulate matter and microbial contaminants. (Bircher *et al*., 2018; Juckers *et al*., 2021; Zhai *et al*., 2004)

Sterile filtrates were frozen at –80 °C and lyophilized using a laboratory freeze dryer for 48 h to preserve the thermolabile bioactive constituents. The resulting dry powder was weighed to determine the extraction yield and reconstituted in sterile distilled water to obtain a stock concentration of 100 mg/mL. Stock solutions were aliquoted and stored at –20 °C until further use (Lorenzo *et al*., 2018; Xi, 2015).

Dung sample

Fresh dung samples were collected from the same animals within two hours of defecation to minimize environmental contamination. Samples were

transported in sterile containers and air-dried under controlled laboratory conditions (25 °C, 40% relative humidity) for 72 h until the moisture content fell below 10% (Bartlett and James, 1980; Seames *et al*., 2002).

The dried dung was pulverized using a sterile mechanical grinder and sieved through a 60-mesh sieve to obtain uniform particles. Methanolic extraction was performed using a 1:10 (w/v) ratio of dung powder to analytical-grade methanol. The mixture was subjected to rotary shaking at 150 rpm for 24 h at room temperature (Bartlett and James, 1980).

After extraction, the mixture was filtered through Whatman No. 1 filter paper. The filtrate was concentrated under reduced pressure using a rotary evaporator at 40 °C to remove the solvent. The dried crude extract was reconstituted in 1% dimethyl sulfoxide (DMSO) to achieve a stock concentration of 100 mg/mL and stored at –20 °C in sterile vials until analysis.

Phytochemical Characterization

Preliminary phytochemical screening was conducted to qualitatively and quantitatively assess the presence of major classes of bioactive compounds, including Phenolic compounds, Flavonoids, alkaloids, and saponins, using standard colorimetric and precipitation assays. Total Phenolic Content (TPC) was determined using the Folin–Ciocalteu method and expressed as mg gallic acid equivalents (GAE) per gram of extract. All measurements were performed in triplicate.

Test Microorganisms and Culture Conditions

Four American Type Culture Collection (ATCC) reference strains were selected based on their clinical and agricultural relevance (Al-Maqtari *et al*., 2020). *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923, *Salmonella Typhimurium* ATCC 14028. Bacterial strains were maintained on nutrient agar slants at 4 °C and subcultured monthly. For antimicrobial assays, fresh overnight cultures were prepared in nutrient broth and incubated at 37 °C for 18–24 hours. Bacterial suspensions were standardized to 0.5 McFarland turbidity (~1.5×10^{-1} CFU/ml) using Spectrophotometric measurement at 625 nm (Jayari *et al*., 2017).

Evaluation of Antimicrobial Activity

Antibacterial activity was assessed using the agar well diffusion method following CLSI standards. Mueller–Hinton agar (MHA) plates were prepared and quality-controlled for pH (7.2–7.4) and agar depth (4 mm). Standardized bacterial inoculum (100 µl) were spread uniformly on MHA plates to form confluent lawns. Wells with a diameter of 6 mm were punched aseptically using a sterile cup borer. Each well was loaded with 50 µl of the test extract (100 mg/ml), positive control (gentamicin, 10 µg/ml), or solvent control (sterile distilled water or 1% DMSO).

Plates were incubated aerobically at 37 °C for 18–24 hours. Zones of inhibition were measured in millimeters using digital calipers. Measurements were taken from three different angles and averaged. All assays were conducted in triplicate (Muneer *et al*., 2017).

Minimum Inhibitory Concentration (MIC) Determination

Minimum inhibitory concentrations (MICs) were determined using the broth micro dilution method in sterile 96-well micro plates in accordance with the Clinical and Laboratory Standards Institute guidelines.

Two-fold serial dilutions of extracts were prepared in cation-adjusted Mueller–Hinton broth to achieve final concentrations ranging from 500 to 0.98 µg/ml. The bacterial suspensions were adjusted to yield a final inoculum concentration of approximately 5 × 10 CFU/ml per well.

Quality control wells included sterility controls (media only), growth controls (bacteria without extract), and positive antibiotic controls (gentamicin; 32–0.06 µg/ml). The plates were incubated at 37 °C for 18–20 h.MIC values were defined as the lowest extract concentration showing complete inhibition of visible bacterial growth. The results were confirmed by measuring the absorbance at 600 nm and sub-culturing aliquots onto Mueller–Hinton agar plates (Fröberg *et al.,* 2023).

Stability Studies

Extract stability was evaluated under two storage conditions: frozen (–20 °C) and ambient room temperature (~25 °C; 40%–60% relative humidity). Approximately 100 mg of each extract was aliquoted into sterile Eppendorf tubes, sealed with parafilm, and stored in triplicate. For frozen storage, the samples were analyzed at 0, 4, 8, and 12 weeks. For room-temperature storage, the samples were evaluated weekly for 4 weeks. At each time point,

antimicrobial activity was assessed using an agar well diffusion assay. The residual activity was expressed as a percentage of the initial activity (time 0 = 100%)(Gupta *et al*., 2016).

Statistical Analysis

All experiments were conducted in triplicate to ensure reproducibility. Data are expressed as mean ± standard deviation (SD). Ninety-five percent confidence intervals (95% CI) were calculated using Student's t-distribution (degrees of freedom = 2). Statistical analyses were performed using R statistical software (version 4.3.0). Statistical significance was set at $p < 0.05$.

RESULTS

Sample Collection

Fresh midstream urine from five healthy Gir cows was sterile-filtered (0.45- and 0.22-µm), lyophilized, and reconstituted to 100 mg/mL stock in water, stored at –20 °C. Fresh dung from the same cows was air-dried, pulverized, methanolic-extracted (1:10 w/v, 24 h), concentrated via rotary evaporation, and reconstituted to 100 mg/ml in 1% DMSO, stored at –20 °C.

Fig. 1. Cow excreta sample Collection

Extract Yield and Phytochemical Characterization

Lyophilization of fresh cow urine yielded 12.3 ± 0.8 g of dry powder per liter, indicating efficient recovery of soluble constituents. Methanolic extraction of air-dried cow dung produced a crude extract yield of 8.7 ± 1.2% (w/w) relative to the starting dry material.

Preliminary phytochemical screening confirmed the presence of multiple bioactive compound classes in both extracts (Table 1). Quantitative analysis revealed that the cow dung extract possessed a significantly higher total phenolic content (78.9 ± 5.1 mg GAE/g extract) than the cow urine extract (45.6 ± 3.2 mg GAE/g extract). Flavonoids were strongly positive in the dung extract and were present in the urine extract, whereas alkaloids were detected in both (weakly positive in urine). Saponin were detected only in the dung extract.

Table 1. Phytochemical Profile of Extracts

Phytochemical Component	Cow Urine Extract	Cow Dung Extract
Total Phenolics (mg GAE/g)	45.6 ± 3.2	78.9 ± 5.1
Flavonoids	Present	Strongly present
Alkaloids	Weak positive	Present
Saponins	Not detected	Present

Antimicrobial Activity

Both extracts exhibited measurable antibacterial activity against all four ATCC reference strains. However, the cow urine extracts consistently produced larger zones of inhibition than the cow dung extracts (Table 2).

The most pronounced difference was observed against *E. coli*, where the cow urine extract produced an inhibition zone that was 19.4% larger than that produced by the dung extract. Overall, the inhibition zones for cow urine ranged from 12.2 to 16.0 mm, whereas those for dung extract ranged from 11.5 to 13.4 mm.

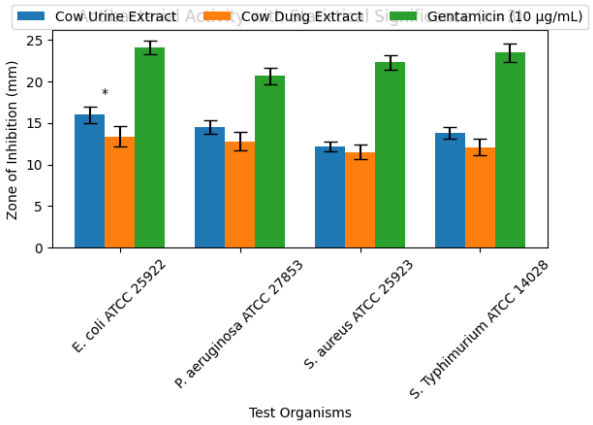

Fig. 2. Zone of Inhibition (mm) ± SD (n = 3)

Gentamicin (10 µg/ml) produced significantly larger zones (20.7–24.1 mm), confirming assay validity and the expected higher potency of

Table 2. Zone of Inhibition (mm) ± SD (n = 3)

Organism	Cow Urine Extract	Cow Dung Extract	Gentamicin (10 ìg/ml)
E. coli ATCC 25922	16.0 ± 1.0	13.4 ± 1.2	24.1 ± 0.8
P. aeruginosa ATCC 27853	14.5 ± 0.8	12.8 ± 1.1	20.7 ± 1.0
S. aureus ATCC 25923	12.2 ± 0.6	11.5 ± 0.9	22.3 ± 0.9
S. Typhimurium ATCC 14028	13.8 ± 0.7	12.1 ± 1.0	23.5 ± 1.1

Table 3. Minimum Inhibitory Concentrations (mg/ml) ± SD (n = 3)

Organism	Cow Urine Extract	Cow Dung Extract	Gentamicin (ìg/mL)
E. coli ATCC 25922	0.25 ± 0.03	0.50 ± 0.06	0.06 ± 0.01
P. aeruginosa ATCC 27853	0.50 ± 0.04	0.75 ± 0.07	0.25 ± 0.02
S. aureus ATCC 25923	0.38 ± 0.04	0.60 ± 0.05	0.12 ± 0.01
S. Typhimurium ATCC 14028	0.47 ± 0.05	0.70 ± 0.06	0.08 ± 0.01

conventional antibiotics.

Minimum Inhibitory Concentration (MIC) Determination

Broth micro dilution assays revealed consistently lower MIC values for cow urine extract than for cow dung extract (Table 3), indicating greater antimicrobial potency.

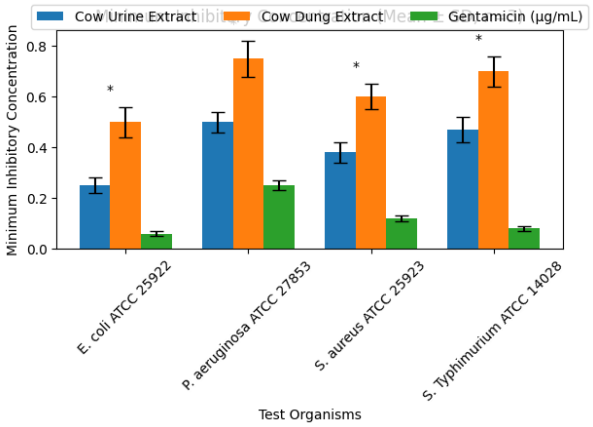

Fig. 3. Minimum Inhibitory Concentrations (mg/ml) ± SD (n = 3)

Cow urine extracts demonstrated 33%–50% lower MIC values relative to dung extract across all organisms tested. The greatest reduction (50%) was observed against *E. coli*. MIC values for cow urine ranged from 0.25–0.50 mg/ml, whereas those for dung extract ranged from 0.50-0.75 mg/ml. As expected, gentamicin exhibited substantially lower MIC values (0.06–0.25 µg/ml), reflecting approximately 500–1000-fold greater potency than crude natural extracts.

Statistical Analysis

All measurements were performed in triplicate, and 95% confidence intervals were calculated. The half-width of 95% confidence intervals for the zone of inhibition measurements ranged from 1.49 to 2.98 mm, whereas the MIC confidence interval half-widths ranged from 0.075 to 0.174 mg/ml. These values indicate acceptable experimental precision and reproducibility for comparative natural product antimicrobial studies.

Stability Studies

Both extracts retained high antimicrobial activity under frozen storage conditions (Table 4).

Table 4. Percentage Activity Retention at –20 °C

Time (weeks)	Urine Extract (%)	Dung Extract (%)
0	100	100
4	99.5	98
8	99	95
12	96	90

After 12 weeks at –20 °C, the cow urine extract retained approximately **96% of its initial activity** (<4% loss), whereas the dung extract retained **90% activity** (~10% loss). These results indicate the strong preservation of bioactivity under freezing conditions.

Stability at Room Temperature (~25 °C)

A more pronounced loss of activity was observed under ambient storage (Table 5).

After four weeks at room temperature, the urine extract retained approximately 85% of its activity (15% loss), whereas the dung extract retained approximately 75% of its activity (25% loss). The mean activity retention over the storage period was approximately 98% for urine samples stored at –20

Table 5. Percentage Activity Retention at Room Temperature

Time (weeks)	Urine Extract (%)	Dung Extract (%)
0	100	100
1	96	90
2	93	85
3	90	80
4	85	75

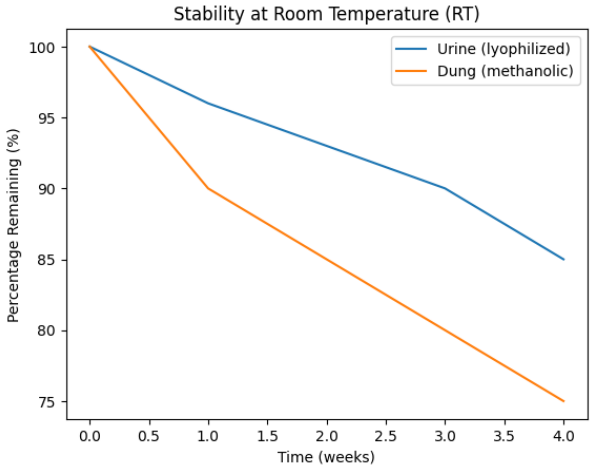

Fig. 4. Percentage Activity Retention at-20 °C

°C and 96% for dung samples under the same frozen conditions. At room temperature (RT), urine samples retained about 92% activity, while dung samples showed slightly lower retention at around 86%. These values highlight the superior stability of both sample types under refrigerated storage compared to ambient conditions.

These findings demonstrate that frozen storage significantly enhances long-term stability, whereas ambient temperature accelerates the degradation of

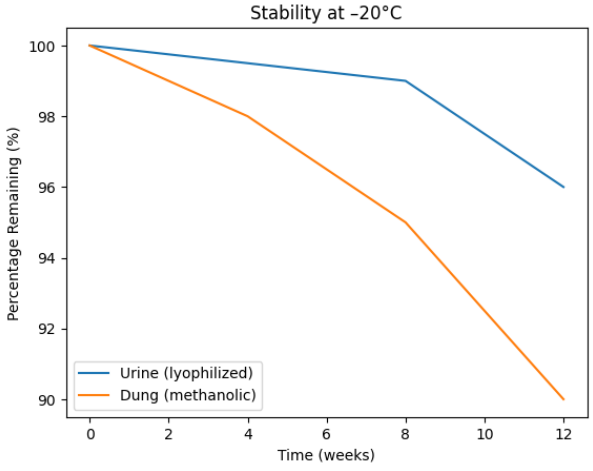

Fig. 5. Stability at Room Temperature (~25 °C)

the bioactive constituents.

DISCUSSION

The present study comparatively evaluated the antimicrobial efficacy and storage stability of lyophilized and Methanolic cow dung extracts derived from organically maintained Gir cows. The findings demonstrated that both extracts exhibited measurable antibacterial activity against clinically significant gram-positive and gram-negative pathogens; however, the cow urine extract consistently exhibited superior potency in all assays.

Phytochemical Composition and Antimicrobial Activity

Phytochemical screening revealed the presence of phenolics, flavonoids, and alkaloids in both extracts, with the cow dung extract exhibiting a higher total phenolic content (78.9 ± 5.1 mg GAE/g) than the cow urine extract (45.6 ± 3.2 mg GAE/g) (Ndezo Bisso *et al*., 2022). Phenolic compounds are widely recognized for their antimicrobial properties, primarily through membrane disruption, enzyme inhibition, and oxidative stress induction. The higher phenolic content in the dung extract is likely attributable to plant-derived secondary metabolites concentrated during digestion and excretion (Kalinowska *et al*., 2024; Laubach *et al*., 2013).

Interestingly, despite its lower total phenolic content, cow urine extract demonstrated significantly greater antimicrobial efficacy in both agar diffusion and MIC assays. This suggests that antimicrobial potency is not solely dependent on total phenolic concentration but may be influenced by the presence of specific bioactive metabolites, peptides, volatile fatty acids, urea derivatives, and low-molecular-weight compounds unique to urine. Synergistic interactions among these constituents may enhance antibacterial effects beyond what would be predicted based on the phenolic content alone (Caponio *et al*., 2022; Taleb *et al*., 2016).

The greater susceptibility of *Escherichia coli* compared to that of other organisms aligns with previous observations that certain gram-negative Enterobacteriaceae are particularly sensitive to natural metabolites that affect membrane integrity. Although gram-negative bacteria possess an outer membrane barrier, small amphiphilic molecules present in the urine extract may facilitate permeabilization. The comparatively moderate activity against *Staphylococcus aureus* suggests

possible differences in cell wall composition or resistance mechanisms influencing extract interactions (Bordean *et al.*, 2023; Simoes *et al.*, 2008).

As expected, gentamicin exhibited substantially lower MIC values (0.06-0.25 ìg/ml), which was approximately 500-1000-fold more potent than the crude extracts. This disparity is anticipated, given that conventional antibiotics are purified and highly concentrated compounds, whereas the tested extracts represent complex crude mixtures. Nonetheless, the observed inhibition zones (12.2–16.0 mm for urine extract) indicate biologically meaningful antimicrobial activity, particularly considering the crude nature of the preparations (Atashbeyk *et al.*, 2014; Wasihun *et al.*, 2023).

Comparative Efficacy of Urine and Dung Extracts

The MIC values of cow urine extract were 33%–50% lower than those of cow dung extract for all tested pathogens. The most pronounced improvement was observed against *E. coli*, with a 50% MIC reduction. This consistent pattern across both the diffusion and dilution assays strengthens the reliability of the comparative findings. (Javed *et al.*, 2022; Veloo *et al.*, 2025)

Although the cow dung extract contained higher phenolic concentrations and detectable saponins, its antimicrobial performance was comparatively weaker. This may reflect differences in compound solubility, extraction efficiency, or the presence of inhibitory matrix components in the dung that limit diffusion in agar or bioavailability in broth systems. Additionally, the methanol extraction process may preferentially isolate phenolics while excluding certain volatile or water-soluble antimicrobial metabolites that are retained in lyophilized urine (Balakrishnan and Kumar Subramanian, 2024; Javed *et al.*, 2022).

Stability and Storage Implications

Stability studies demonstrated that both extracts retained high antimicrobial activity under frozen storage (–20 °C), with the urine extract showing <4% loss and the dung extract showing ~10% loss over 12 weeks. These results confirm the effectiveness of lyophilization and low-temperature storage in preserving thermo labile bioactive compounds. Freeze–drying likely reduces hydrolytic and oxidative degradation by minimizing residual moisture and enzymatic activity (Manohar and Ramesh, 2019; Passot *et al.*, 2007).

In contrast, room-temperature storage resulted in notable reductions in activity within four weeks (15% loss for urine extract and 25% loss for dung extract). The accelerated decline under ambient conditions suggests that thermal degradation, oxidation, and possible moisture-mediated reactions affect the bioactive constituents. The greater instability of the dung extract may be associated with phenolic oxidation or interactions between multiple compound classes (Howard *et al.*, 2012; Javadi *et al.*, 2021).

From an application standpoint, these findings highlight the importance of controlled storage conditions to maintain efficacy in potential pharmaceutical, agricultural, or biopreservative formulations. Frozen storage appears to be optimal for long-term preservation, whereas room-temperature formulations may require stabilizing agents or protective packaging.

Implications for Sustainable Antimicrobial Development

This study contributes to the growing body of research on sustainable waste valorization and natural antimicrobial development. Utilizing bovine-derived waste materials aligns with circular economy principles by transforming agricultural byproducts into value-added bioactive products. Such approaches may reduce the environmental burden while offering low-cost antimicrobial alternatives for veterinary, agricultural, or surface-disinfection applications.

CONCLUSION

The present study demonstrated that both lyophilized cow urine extract and Methanolic cow dung extract possess measurable in vitro antimicrobial activity against clinically relevant gram-positive and gram-negative ATCC reference strains. Among the two, cow urine extract consistently exhibited superior antibacterial efficacy, as evidenced by larger zones of inhibition and 33-50% lower MIC values across all tested organisms. The strongest activity was observed against *Escherichia coli*, highlighting its potential relevance against enteric pathogens.

Although cow dung extract contained a higher total phenolic content, antimicrobial potency was greater in the cow urine extract, suggesting that specific bioactive metabolites and synergistic interactions, rather than the total phenolic concentration alone, play a critical role in

antibacterial activity. These findings underscore the importance of comprehensive bioactive profiling beyond quantitative phytochemical estimation.

Stability studies further revealed that both extracts retained substantial antimicrobial activity under frozen storage (–20 °C) for up to 12 weeks, with minimal activity loss (<10%). In contrast, room temperature storage resulted in moderate reductions in efficacy (15–25%) within four weeks, emphasizing the necessity of controlled storage conditions to preserve bioactivity.

Overall, these results support the potential of bovine-derived bioactive extracts, particularly lyophilized cow urine extracts, as sustainable and eco-friendly antimicrobial agents. This study contributes to the valorization of agricultural waste materials and provides foundational evidence for the development of natural antimicrobial formulations. Future studies focusing on compound isolation, mechanistic elucidation, toxicity assessment, and evaluation of multidrug-resistant clinical isolates are warranted to advance their translational applications.

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Contribution of the author

Bipin Patel came up with the idea, did the literature search and data analysis and developed the final draft, other author contributed for review and technical inputs.

Conflict of Interest - None

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