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Original Research Article

Pharmacological potential of cow urine from Red Sindhi breed: *In-vitro* study on antimicrobial and cytoprotective properties

Nikita Paliya[1], Chetana Deoghare[2*]

[1]Dept. of Clinical Research & Healthcare Management, Institute of Sciences, Humanities & Liberal Studies, Indus University, Rancharda, Ahmedabad, Gujarat, India

[2]The School of Sciences, P P Savani University, Kosamba, Surat, Gujarat, India

Abstract

Background: Cow urine, a central component in traditional Ayurvedic medicine, is credited with therapeutic benefits for various ailments. Recent research has emphasized the importance of breed-specific studies, but most findings are limited to only a few breeds. This study investigates the pharmacological efficacy of urine from the Red Sindhi breed, evaluating its antimicrobial and cytoprotective properties.

Materials and Methods: Urine samples were collected from adult, pregnant, lactating, and calf Red Sindhi cows under aseptic conditions, filtered, and distilled. The Cow urine samples' antimicrobial activity was tested against two Gram-positive (*Bacillus subtilis*, *Staphylococcus aureus*) and two Gram-negative bacteria (*Klebsiella pneumoniae*, *Proteus vulgaris*) using well diffusion assays. Cytotoxicity and cell viability in human normal fibroblast and HepG2 cells were assessed through MTT assays, while mitochondrial membrane potential (MMP), mitochondrial mass (MM), and reactive oxygen species (ROS) were measured for oxidative stress assessment.

Results: Raw and distilled cow urine (ARCU, ADCU) from adult Red Sindhi exhibited antibacterial activity, particularly against Proteus vulgaris and Bacillus subtilis, comparable to chloramphenicol. MTT assays showed no significant cytotoxicity, with both ARCU and ADCU supporting cell viability in fibroblast and HepG2 cells. Antioxidant analysis indicated enhanced MMP, MM, and reduced ROS levels in treated cells, suggesting protective effects against oxidative stress.

Conclusions: The study demonstrates that Red Sindhi cow urine possesses notable antibacterial, and cytoprotective activities. These findings underscore the potential of Red Sindhi cow urine as a therapeutic agent, supporting its traditional use in Ayurveda and encouraging further studies on breed-specific efficacy.

Keywords: Cow urine, Red Sindhi breed, Antimicrobial activity, Antioxidant activity, Cryoprotection, Oxidative stress, Ayurveda.

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1. Introduction

Cows have held a central role in Indian culture and economy for centuries. Both the Charaka and Sushruta Samhitas described the medicinal uses of cow urine, crediting it with the ability to treat a wide range of ailments such as vitiligo, eye diseases, cough, jaundice, and asthma. Today, cow urine remains an important element of Ayurvedic medicine, though its efficacy has often gone unverified due to a lack of scientific studies. Most research has focused on a few indigenous cow breeds, such as the Gir breed, whose urine has demonstrated antioxidant and antibacterial activity.[1] Additionally, urine from the Deoni breed has shown hepatoprotective and wound-healing properties.[2]

Recent studies have explored the benefits of cow urine distillate, finding it effective in various health applications, including antibacterial, antifungal, antihepatotoxic, and immunomodulatory properties.[3] Panchgavya, a traditional Ayurvedic formulation made from cow milk, curd, ghee, urine, and dung, has also been shown to treat various conditions such as jaundice, fever, and cognitive decline.[4] The antioxidant and free radical-scavenging properties of Panchgavya have further emphasized its medicinal potential.

Cows and other herbivores get a lot of bioactive chemicals from the plants they eat. Phytochemicals, including terpenoids, phenolics, and alkaloids, have been scientifically shown to influence animal metabolism

*Corresponding author: Chetana Deoghare
Email: chetskm@gmail.com

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positively, offering protection against conditions such as cancer, heart disease, diabetes, and infections.[5] This connection between plant-based diets and the pharmacological activities of urine highlights the potential of cow urine in medical treatments.

Despite the evidence supporting the therapeutic benefits of cow urine, research has been conducted on only two out of the 41 recognized indigenous cow breeds in India. The majority of studies fail to specify the breed from which urine samples are sourced. However, research from a few indigenous breeds suggests that breed-specific factors may influence the therapeutic value of cow urine.[6] Given this context, it is crucial to explore the therapeutic properties of cow urine more systematically, focusing on indigenous breeds like the Red Sindhi. To better understand the potential health benefits of cow urine, comprehensive in vitro study was conducted on urine from adult, pregnant, lactating, and calf Red Sindhi cows. These studies aimed to investigate antimicrobial, Cytotoxicity and cell viability in human normal fibroblast and MTT assays, mitochondrial membrane potential, mitochondrial mass and reactive oxygen species assays, providing valuable insights into the pharmacological efficacy of cow urine.

2. Materials and Methods

Urine samples were collected from various stages of adult, pregnant, lactating, and calf of Red Sindhi cow for different analyses. For the purpose of this study, a mature cow (three years old) was housed in Bellippadi, a village in Puttur Taluk, Dakshina Kannada, Karnataka, India (79° 33' 11" E, 13° 26' 30" N) since 2012. The cow was kept in a traditional barn with a mud floor lined with green leaves for bedding. When the cow turned four, it gave birth to a female calf through natural breeding methods. The lactation period lasted for nine months, after which the cow entered its dry phase. The calf was raised alongside its mother. The cow was fed green grass and paddy straw, while receiving veterinary care throughout the study. During the lactation period, the cow was also provided with *maddi* (a powdered mixture of horse gram *chunni*, broken rice, oil cake, and rice flour). The lactating calf was fed chopped green grass and paddy straw.

Early morning urine (100-150 mL) was collected under aseptic conditions from adult (three years old), pregnant (eight months pregnant), lactating (one month after birth), and calf (one month old) Red Sindhi cows. The samples were filtered using a 0.2 µm syringe filter, and part of the samples was distilled at 100°C using a specially designed glass distillation apparatus. For ease of reference, the samples were assigned distinct names and abbreviations. All urine samples were stored at -18°C for further analysis.

The antibacterial properties of raw and distilled urine (ARCU and ADCU) from adult cows were assessed using two Gram-positive (*Bacillus subtilis, Staphylococcus aureus*) and two Gram-negative strains (*Klebsiella pneumoniae*, *Proteus vulgaris*). A well diffusion assay was conducted, with zones of inhibition measured after 24hours.

MTT assays were performed to evaluate the cytotoxicity of ARCU and ADCU on human normal fibroblasts and HepG2 cell lines. Cells were treated with varying concentrations of urine (0.2%, 0.4%, 0.8%, etc.) for 24 and 48 hours. Viability was calculated by measuring absorbance at 550 nm.

2.1. Statistical analysis

All data were analyzed using one-way ANOVA followed by Tukey's or Dunnett's post-hoc tests. Results were reported as mean ± SEM or mean ± SD with significance levels set at $p < 0.05$, $p < 0.01$, and $p < 0.001$. All experiments were conducted in triplicate (n=3).

3. Results

As per the modern pharmacology, cow urine is useful for numerous diseases A number of ailments could be treated and this therapy is being used even for dreaded diseases like cancer, AIDS, diabetes, and skin problems. It is antibacterial, antifungal, antiviral, antineoplastic (anticancer), anticonvulsive, antispasmodic, and non-toxic.

The effects of raw and distilled cow urine from an adult Red Sindhi (ARCU, ADCU) on bacterial strains are studied and zone of inhibition is shown below (**Table 1**). Chloramphenicol effectively inhibited the growth of *Bacillus subtilis* (14.8 mm), *Staphylococcus aureus* (9.2 mm), *Klebsiella pneumoniae* (12.8 mm), and Proteus vulgaris (17.9 mm). ARCU and ADCU inhibited the growth of Gram-positive bacteria as follows: *Bacillus subtilis* (13.1 mm; 11.30 mm) and *Staphylococcus aureus* (6.8 mm; 4.7± mm). ARCU and ADCU also inhibited the growth of Gram-negative bacteria as *Klebsiella pneumoniae* (11.3 mm; 10.8 mm) and *Proteus vulgaris* (16.7 mm; 14.3 mm), respectively. Raw cow urine sample shows better antibacterial activity as compared to the distilled cow urine sample.

Table 1: Antibacterial activity (mm) of cow urine samples from adult Red Sindhi

Treatment sample (100 µg/mL)	*Bacillus subtilis*	*Staphylococcus aureus*	*Klebsiella pneumoniae*	*Proteus vulgaris*
Chloramphenicol	14.8±0.9	9.2± 0.6	12.8±1.1	17.9±0.6
ARCU	13.1±0.6[a]	6.8± 1.1[a]	11.3±0.6[#]	16.7±0.9[a]
ADCU	11.3±0.6[a]	4.7± 0.6[a]	10.8±1.1[#]	14.3±0.6[a]

(Mean ± SEM) (n=3)

A quick glance at **Table 2,** mitomycin-c treatment led to reduce cell viability (55.32%), compared to the control (100%). The treatment with different cow urine concentrations (0.2, 0.4, 0.8, 1.6, 3.2, and 6.4 µM) of ARCU and ADCU showed no significant decrease in cell viability compared to control cells. There was a slight, insignificant increase in viability at 0.4 µM concentration of ARCU (98.67%) and ADCU (110.47%) in human normal fibroblasts at 24h.

Table 2: Cell viability (%) in human normal fibroblasts treated with cow urine

Treatment Sample	24h	48h
Control	100.00±0.2	100.00±0.8
Mitomycin-C (30 µM)	55.32±1.1	34.12±0.9
	ARCU	ADCU
0.2	91.45±0.2	97.63±0.9
0.4	98.67±0.4	110.47±0.8
0.8	94.86±0.6	107.29±0.3
1.6	94.55±0.7	106.72±0.3
3.2	93.48±1.1	106.20±0.5
6.4	91.73±0.1	98.42 ±1.01

(Mean ± SEM) (n=3)

Table 3: Cell viability (%) in HepG2 cell line treated with cow urine

Treatment sample	24h	48h
Control	100.00±0.3	100±0.4
Mitomycin-C (30 µM)	69.22±0.4	54.85±0.8
	ARCU	ADCU
0.2	92.18±0.7	96.50±1.6
0.4	108.45±0.3	109.54±0.1
0.8	100.12±0.8	108.51±0.5
1.6	99.80±0.2	97.75±0.9
3.2	96.55±0.1	97.70±2.1
6.4	94.62±0.1	97.44±1.6

(Mean ± SEM) (n=3)

As presented in **Table 3,** cell viability decreased to 69.22% in mitomycin-c treated HepG2 cells at 24h. The treatment with 0.2 µM concentration of ARCU (92.18%) and ADCU (96.50%) showed no significant difference in viability compared to the control.

At 0.4 µM concentration of ARCU (108.45%) and ADCU (109.54%), there was an observed increase in viability. Higher concentrations did not cause significant cell death. Similar results were noted at 48h, indicating that the concentrations employed were not cytotoxic.

As shown in **Table 4,** the MFI of control cells was 786.22 at 24h. ARCU-treated fibroblasts showed MFI values of 780.14 at 0.1% concentration and 795.38 at 0.2% concentration in 24h, which were not significantly different from control cells. ARCU treatment at 48h significantly increased MFI (1103.6 for 0.1% concentration and 1114.67 for 0.2%) compared to the control i.e.1010.87. ADCU-treated cells also showed increased MFI for 0.1% (860.35 in 24h; 1012.80 in 48h) and 0.2% (923.10 in 24h; 1012.65 in 48h).

MM: When compared to the control (668.12 in 24 h; 1015.53 in 48 h), looking to results both ARCU and ADCU treatments increased the MM. ARCU and ADCU exhibited (683.05; 692.25 for 24h) and (1018.65; 1021.05, 48h) for 0.1% concentrations, and (692.25; 728.25, 24h) (1021.45; 1050.75, 48h) for 0.2% concentrations.

ROS: Control cells showed an MFI of 171.15 (24h) and 312.25 (48h). ARCU and ADCU treatments at 0.1% resulted in minor increases at 24h (174.62; 177.75) but reduced ROS levels at 48h (288.30; 278.15). At 0.2%, ARCU and ADCU exhibited MFI values of 106.50; 167.80 at 24h, and 272.85; 268.70 at 48h, showing decreased ROS levels.

Table 4: MMP, MM and ROS in human normal fibroblasts treated with cow urine

Treatment Sample	MMP (24h)	MMP (48h)	MM (24h)	MM (48h)	ROS (24h)	ROS (48h)
Control	786.22±2.10	1010.87±2.50	668.12±3.25	1015.53±3.50	171.15±2.50	312.25±4.80
ARCU (0.1%)	780.14±5.45	1103.67±6.22	683.05±7.78	1018.65±5.45	174.62±3.45	288.30±1.20
ARCU (0.2%)	795.38±8.30	1114.67±6.80	692.25±5.65	1021.45±2.80	106.50±2.87	272.85±1.15
ADCU (0.1%)	860.35±4.25	1012.80±7.20	710.35±8.50	1021.05±4.10	177.75±2.95	278.15±1.75
ADCU (0.2%)	923.10±9.50	1012.65±5.95	728.25±5.70	1050.75±8.25	167.80±4.25	268.70±1.85

MMP: In **Table 5**, ARCU and ADCU treatments at 0.1% (275.60 ± 2.10; 300.40 ± 12.20, 24h) (180.90 ± 3.20; 215.80 ± 3.10, 48 h) and 0.2% (284.80±1.40; 310.70±2.20, 24h) (195.10±2.80; 215.70±2.60, 48h) led to a decrease in MMP when compared to the control (590.30±21.70 at 24 h; 340.50 ± 4.90 at 48h).

MM: ARCU and ADCU treatments at 0.1% and 0.2% resulted in increased MM. ARCU and ADCU at 0.1% showed MFI of 800.70 ± 6.10 and 810.40 ± 5.80 (24 h), and 665.50 ± 3.90 and 715.70 ± 3.80 (48 h). At 0.2%, MFI values were 845.90 ± 6.80 and 830.50 ± 6.30 (24 h), and 695.20 ± 9.60 and 730.50 ± 5.70 (48h).

Table 5: MMP, MM and ROS in HepG2 cell line treated with cow urine

Treatment Sample	MMP (24h)	MMP (48h)	MM (24h)	MM (48h)	ROS (24h)	ROS (48h)
Control	590.30±21.65	335.45±5.25	636.40±11.30	634.28±7.85	203.35±1.95	278.30±1.45
ARCU (0.1%)	275.15±1.54	183.18±2.22	800.45±4.85	668.15±3.90	49.58±0.60	54.45±1.35
ARCU (0.2%)	283.75±1.10	195.50±1.10	836.25±4.50	689.90±8.25	49.50±0.85	52.65±0.30
ADCU (0.1%)	299.18±10.45	209.50±2.55	807.40±4.90	704.10±3.75	48.75±0.58	55.65±0.30
ADCU (0.2%)	307.10±1.85	211.75±2.65	820.75±4.85	719.45±3.30	49.75±0.58	53.85±0.30

ROS: ARCU and ADCU treatments at 0.1% and 0.2% concentrations showed decreased ROS levels at 24 h and 48 h. ARCU and ADCU respectively showed MFI of 52.20 ± 1.50 and 51.80 ± 0.90 (24 h); 56.10 ± 1.60 and 57.90 ± 0.60 (48h) for 0.1% concentrations. At 0.2%, the MFI was 51.20 ± 0.70 and 51.90 ± 0.50 (24h), and 54.60 ± 0.70 and 55.80 ± 0.50 (48h).

4. Discussion

Cow urine has been used in traditional medicine for centuries to treat various ailments, particularly in the Indian subcontinent. Despite its frequent use in folk remedies, there has been limited scientific investigation into the therapeutic potential of cow urine from indigenous breeds like the Red Sindhi, an indigenous cattle breed from Karnataka, India. The current study sought to fill this gap by evaluating the pharmacological activities of both Raw and distilled cow urine (ARCU, ADCU) from adult Red Sindhi, breed.

Urine analysis is a valuable tool in veterinary science, providing insights into the health and metabolic status of animals. In this study, the first objective was to determine whether any pathological changes could be detected in the urine samples. No abnormalities were found in the physical, chemical, or microscopic examinations of urine from adult, pregnant, lactating, or calf Red Sindhi cows. The results align with findings from previous studies, which suggest that urine from healthy animals typically lacks pathological markers.[1] The absence of albumin, glucose, bile salts, bile pigments, and reducing sugars, combined with alkaline pH values, further indicated the healthy status of the cows used in this study.[8] The alkaline nature of the urine in all samples (with pH values ranging from 7.4 to 8.4) is consistent with findings in cattle, where the breakdown of urea in the urinary tract can release ammonia, causing an alkaline shift. This alkaline environment is typical in herbivores due to their plant-based diets, which influence both the pH and the composition of urinary excretions.[9]

Significant variations in the concentrations of biochemical constituents, such as urea, urea nitrogen, uric acid, creatinine, calcium, sodium, chloride, and total phenol, were observed across the different groups of urine samples. These differences are reflective of the physiological changes occurring in different life stages (pregnancy, lactation) and are also affected by dietary intake and metabolic requirements.[10]

MMP is nothing but electrochemical potential composed of transmembrane electrical potential and proton gradient. This is responsible for maintenance of energy and synthesis of ATP. In human normal fibroblasts 0.1% and 0.2% concentration of ARCU showed significant ($P < 0.01$) increase in MMP only at 48 h. A significant increase in MMP was observed by both the treatments of ADCU, exhibiting $P < 0.05$ for 0.1% and $P < 0.01$ for 0.2% concentration at 24 h duration. However, an insignificant decrease in MMP was shown by HepG2 cell line with cow urine ARCU and ADCU (0.1 and 0.2%) treatments. This signifies a very positive effects of cow urine only with specific tissue probably in a dose dependent manner. Decrease in the MMP attributes to mitochondrial dysfunction followed by cell injury and this is an early sign of apoptosis.[11]

Cardiolipin present in mitochondrial inner membrane, found to bind with cytochrome c and oxidation of cardiolipin releases cytochrome c from mitochondrial membrane. The dye NAO binds to the cardiolipin and measures the mitochondrial mass.[12,13] The decreased MMP and severe cardiolipin oxidation releases cytochrome c to the cytoplasm and thereby activates caspase - 9 followed by activation of downstream caspases. The cleavage of effector proteins, including PARP by activated caspase 3 brings DNA fragmentation and leading to cell death.[14] This is the indicator of damage to the mitochondrial membrane and mitochondrial disfunction. An insignificant increase in MM was also observed in human normal fibroblasts treated with ARCU and ADCU (0.1% and 0.2%) at 24h and 48h. However, there was highly significant ($P < 0.001$) increase in MM of HepG2 cell line treated with ARCU (0.1 and 0.2%) at 24h. The ARCU (0.1 and 0.2%) significantly ($P < 0.05$; $P < 0.01$) increased MM at 48h. Also, MM increased significantly ($P < 0.001$) after treatmentwith ADCU (0.1 and 0.2%) at 24h and 48h. Therefore, the treatment with ARCU and ADCU(0.1% and 0.2%) in human normal fibroblasts and HepG2 cell line indicate that there is no mitochondrial damage even at 24h and 48h treatments.[14]

Results of ROS overproduction brings oxidative damage to biomolecules and ROS has the ability to activate mitochondrial intrinsic apoptotic cascades by inhibition of anti- apoptotic protein Bcl-2 and activation and translocation of pro-apoptotic protein Bax to the outer mitochondrial membrane attributed to formation of oligomers).[14,15] This phenomenon is important for permeability transition pore (PTP) formation and release of cytochrome c. Studies also reported that, ROS has the ability to activate p^{38} MAPK

thereby activates and translocates Bax to the outer mitochondrial membrane.[16,17] The ROS level decreased insignificantly in human normal fibroblasts treated with ARCU and ADCU (0.1 and 0.2%) at 48 h. Besides, ROS level was significantly ($P < 0.001$) decreased in HepG2 cell line treated with ARCU and ADCU (0.1 and 0.2%). Thisindicates lack of oxidative damage and ROS mediated apoptosis.

The flow cytometry is used to measure the DNA content in cells. An intercalating dye propidium iodide which binds to DNA and double stranded RNA is commonly used today for this purpose. The results of cell cycle analysis infer those treatments with ARCU and ADCU (0.1 and 0.2%) at 24 h and 48 h duration did not show apoptosis in human normal fibroblastsand HepG2 cell lines.[16]

There was an insignificant increase in S phase was observed in human normal fibroblasts at 24 h and 48 h with ARCU and ADCU treatments. ARCU (0.1 and 0.2%) respectively showed significant ($P < 0.05$, $P < 0.01$) decrease in G_0/G_1 phase at 48h. The ADCU (0.2%) showed significant ($P < 0.05$) decrease in G_0/G_1 phase at 48h. The G_2/M phase was not affected much by treatment with ARCU and ADCU when compared to the control in human normal fibroblasts. However, in HepG2 cell line, treatment with 0.2% of ARCU only showed significant ($P < 0.05$) increase in S phase at 24h duration. Treatment with ADCU (0.1 and 0.2%) respectively showed a significant ($P < 0.05$, $P < 0.01$) increase in S phase only at 24 h. The G_2/M phase was not affected much by treatment with ARCU (0.1%) and ADCU (0.1 and 0.2%). Whereas, ARCU (0.2%) at 24h and 48h respectively showed significant ($P < 0.01$, $P < 0.05$) increase in G_2/M phase in HepG2 cell line. Indicating, ARCU and ADCU did not have an effect on cell cycle.[17]

The pre-treatment with redistilled cow urine showing significant ($P < 0.0001$) anti-clastogenic and anti-genotoxic effect in manganese dioxide and hexavalent chromium treated human peripheral lymphocytes. The significant ($P < 0.001$; $P < 0.0001$) reduction in manganese dioxide and hexavalent chromium induced micronuclei was also observed in human peripheral lymphocytes treated with redistilled cow urine. According to authors, the anticlastogenic and antigenotoxic effect is mainly because of ROS scavenging ability of redistilled cow urine. Krishnamurthi et al. have observed the, ability of redistilled cow urine to overcome the actinomycin-D and hydrogen peroxide induced DNA damage more efficiently than cow urine distillate in human polymorphonuclear leucocytes.[18] They reported, antigenotoxic activity is mainly due to volatile fatty acids and total antioxidant activity of cow urine. This is also evident in the present study. Wherein, the ROS levels were markedly decreased in ARCU and ADCU treated human normal fibroblasts and HepG2 cell line.

5. Conclusions

To investigate the breed-specific therapeutic potential, a comprehensive in vitro and in vivo evaluation was conducted on the pharmacological activities of both raw and distilled urine from adult (ARCU, ADCU), pregnant (PRCU, PDCU), lactating (LRCU, LDCU), and calf (CRCU, CDCU) Red Sindhi cows, an indigenous breed from Karnataka. The study examined antimicrobial, cytotoxic, hepatoprotective, antidiabetic, immunomodulatory, and anti-inflammatory properties. This study offers a more comprehensive evaluation of the pharmacological efficacy of raw and distilled cow urine from a limited number of indigenous breeds. The results confirm the breed-specific therapeutic potential of cow urine and cow urine in its raw form is more beneficial for threptic purposes as compared to the distilled cow urine.

However, further research is required to substantiate these findings through the isolation, characterization, and screening of bioactive compounds within the urine. Future studies could prioritize freely grazing cows that consume natural flora. Additionally, similar studies could be extended to other important Indian cow breeds and crossbreeds to explore potential variations in therapeutic efficacy across breeds. In addition, ARCU (100 µL) showed better antibacterial activity over ADCU against selected Gram positive (*B. subtilis* and *S. aureus*) and Gram-negative bacteria (*P. vulgaris* and *K. pneumoniae*). However, ARCU and ADCU did not show significant effect on cytotoxicity and mitochondrial functions as revealed by their cell viability, MMP, MM, ROS and cell cycle analysis.

6. Source of Funding

None.

7. Conflict of Interest

None.

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