The Haryana Veterinarian (June, 2025) **64(1),** 86-90 ISSN 0033-4359 (P) **Research Article**

OPTIMIZATION OF COW URINE BASED FORMULATION FOR WOUND HEALING APPLICATION

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ABSTRACT

In the present study, formulations based on cow urine were developed for wound healing applications. The bioactive constituents in cow urine and its distillate provide a supportive microenvironment at injury sites, fostering tissue regeneration and aiding functional recovery. Essential elements such as Ca, Mg, Mn, Cu, Zn, and Fe were measured at concentrations of 0.02725±0.004, 0.03650±0.010, 0.85192±0.053, 0.00417±0.002, 0.15983±0.015, and 0.12958±0.009 mg/L, respectively. Notably, cow urine double distillate from the late lactation stage showed a 43.7% inhibition of DPPH, demonstrating significant antioxidant activity. Furthermore, FTIR analysis of cow urine from various lactation stages identified functional groups like hydroxyl, epoxy, epoxide, imine, and carboxylic, underscoring its therapeutic potential for wound healing. The formulations, combining urine distillate with mineral oil or cow ghee and other surfactants, displayed desirable consistency and stability, remaining uncontaminated over a six-month storage period.The comprehensive analysis of mineral composition, FTIR-identified functional groups, antioxidant activity, and formulation stability demonstrated the potential of cow urine-based formulations as effective wound healing agents, offering a strong foundation for future research and promising wound care applications.

Keywords: Antioxidant, Cow urine, Mineral, Wound healing

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Natural products and by-products of both animal and plant origin are renowned for their wound-healing properties, often attributed to their unique bioactive compounds that support multiple healing mechanisms. Cow urine, widely used in traditional medicine, exhibits anti-inflammatory, antimicrobial and antioxidant properties that aid in wound healing by reducing infection risk and promoting cellular repair (Khanuja *et al.*, 2002). Honey, a by-product of bees, combines antimicrobial action with a moist wound environment and promotes tissue regeneration, making it effective for chronic wounds and burns (Molan, 2001). Plant-derived compounds like turmeric and neem, both rich in antioxidants and antimicrobial agents, reduce inflammation and prevent bacterial growth in wounds, which supports faster recovery (Ahmad *et al.*, 2012). Sunflower oil, Saijana root bark extract and Olive oil are quite effective in enhancement of healing process of surgical wound on topical application (Anand *et al.*, 2021). These natural agents, with their diverse properties, act synergistically to enhance wound healing by minimizing infection, reducing inflammation, and stimulating tissue regeneration, making them valuable resources for wound care and therapeutic applications (Tobriya, 2014).

Ancient Indian Ayurvedic texts, such as the Charaka Samhita and Sushruta Samhita, highlight cow urine, or Gaumutra, as a highly effective animal-origin by-product with numerous therapeutic properties. Revered as holy in India, cow urine is non-toxic and has been used in ayurvedic formulations for various health conditions (Sagnik and Palbag, 2018). The by-products of cow urine distillate contain phenolic and flavonoid compounds is a panacea of several diseases (Pathak and Kumar, 2003). Recognizing its potential, recent scientific studies have demonstrated its functional properties, including antioxidant, antibacterial, antifungal, immune-modulating, anticancer, antiseptic, anthelmintic, and bio-enhancing effects. Notably, its medicinal properties-such as bio-enhancer, antibiotic, antifungal, and anticancer-are patented under US patents 6,896,907 and 6,410,059 (Khanuja *et al.*, 2002).

In terms of wound healing, cow urine has shown promising results. External application has been found to accelerate wound healing (Sanganal *et al.*, 2011), and histo-morphological analysis of wounds in goats treated with cow urine revealed increased polymorphonuclear cell infiltration, neovascularization, and fibroblast proliferation (Mishra *et al.*, 2009). Additionally, a study on diabetic Wistar albino rats demonstrated significant wound closure and increased granulation tissue and hydroxyproline content in wounds treated with cow urine distillate (Hirapara *et al.*, 2016). Encouraged by these promising results, this study aimed to optimize a cow urine-based formulation specifically for wound healing applications.

MATERIAL AND METHODS

Collection and Distillation of Cow Urine:

Indigenous Deoni cows housed at the Livestock Research Centre of the National Dairy Research Institute in Bangalore, Karnataka, India (latitude: 12.972442, longitude: 77.580643), were selected for this study. These cows were maintained under uniform farm conditions, stall-fed with seasonal green fodder (hybrid napier grass), dry fodder (ragi straw), and a mineral mixture concentrate from the Karnataka Milk Federation.

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Urine samples were collected mid-stream by the free-catch method in clean containers during early morning hours. A total of 169 samples were obtained from cows across various lactation stages: early (56 samples), mid (41 samples), late (34 samples), and non-lactating (38 samples). Each sample was filtered through sterile filter paper within 1-2 hours of collection and screened for health parameters using "Medi Test Combi 10-Vet" dip sticks. Of these, 154 samples met the health criteria and were used for further analysis.

The samples from each lactation stage were pooled separately and stored in sterile containers at -20°C. Pooled samples underwent a two-step distillation process: following the first distillation, the pH of the distillate was adjusted to 4.0 to eliminate residual ammonia, after which a second distillation was performed in order to reduce the ammonia content, lowered pH minimizes the bacterial growth and enhances antibacterial activity. The final distillates were then stored at -20° C.

Compositional Analysis Using Atomic Absorption Spectrophotometry (AAS):

The levels of six essential minerals - calcium (Ca), magnesium (Mg), manganese (Mn), copper (Cu), zinc (Zn), and iron (Fe) were measured using an optical double-beam atomic absorption spectrophotometer (AAS Model Shimadzu-7880, manufactured by Toshniwal Instruments Pvt. Ltd., India). For analysis, 1 ml of each urine sample was digested in a Kjeldahl flask with 9 ml of a tri-acid mixture comprising nitric acid (HNO_3), perchloric acid ($HClO_4$) and sulfuric acid (H_2SO_4) in a 3:2:1 ratio. The digested samples were then diluted with distilled water, filtered through Whatman filter paper No. 42, and the final volume was adjusted to 25 ml.

AAS-grade standard solutions for calibration were sourced from Merck Life Sciences Pvt. Ltd. Each sample's mineral content was measured using an optical double-beam AAS equipped with an 8-hollow cathode lamp on a motorized turret. Calibration curves for each mineral element were generated using blank and standard solutions of the respective minerals.

Functional Group Analysis Using Fourier Transform Infrared Spectroscopy (FTIR):

The functional groups in cow urine and its distillates from Deoni breed cows at different lactation stages were analysed using Fourier Transform Infrared Spectroscopy (FTIR) with a Shimadzu FTIR instrument and data processing unit. Freeze-dried cow urine samples were mixed with IR-grade potassium bromide (KBr) to form pellets under pressure, creating a thin film for analysis. FTIR is very powerful tool for identifying the type of chemical bond (functional group) in the bioactive compounds present in the cow urine samples. It is quite rapid, accurate and relatively sensitive technique (Jaggi and Vij, 2006). Infrared transmittance spectra were recorded over a wavenumber range of 400 to 4000 cm^{-1}. The obtained spectral data were then compared with reference spectra to identify the functional groups and chemical bonds present in the samples.

Anti-oxidative evaluation by DPPH Radical Scavenging Activity:

The antioxidant activity of cow urine was assessed through the DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging assay following the method described by Valko *et al.* (2007). A standard curve was prepared using Trolox at concentrations ranging from 10 to 80 µM. To prepare the standard reaction, 150 µL of the Trolox solution was added to 150 µL of DPPH solution (0.2 mM), mixed for 5 seconds, and incubated for 30 minutes at 37°C in an ELISA plate reader. The absorbance was then measured at 517 nm against a blank, where Trolox was replaced with double-distilled water. The standard curve plotted % inhibition on the Y-axis against Trolox concentration on the X-axis.

The DPPH radical scavenging activity (% inhibition) was calculated as follows:

$$\%\ \text{DPPH radical scavenging activity} = \frac{\text{absorbance - absorbance}}{\text{absorbance}} \times 100$$

This method was applied to each sample, replacing Trolox with the test sample in the reaction. Based on the % inhibition of absorbance for each sample, the Trolox Equivalent Antioxidant Capacity (TEAC) was determined from the standard curve, and results were expressed as mMTrolox equivalents per µL of urine.

Synthesis of Cow Urine-Based Formulations:

Four ointment formulations were standardized for wound healing applications. The composition of each formulation is outlined in table 1.

Formulation I: Polyvinyl alcohol (PVA) was combined with 100 ml of cow urine double distillate and heated to 100°C with continuous stirring at 3,500 rpm using a magnetic stirrer for 1 hour. After this, 2 g of gelatin was added, and the mixture was stirred at 70°C at 100 rpm for another hour. Physical cross-linking of PVA and gelatin was achieved through freeze-thaw cycles: the mixture was kept at -20°C for 1 hour, followed by room temperature for 40 minutes. This cycle was repeated twice to obtain a gel-like consistency.

Formulation II: All ingredients, except freeze-dried cow urine, were combined and heated in a double boiler. The cow urine was freeze-dried separately to concentrate its components without evaporation loss, then mixed into the melted ingredients.

Formulation III: Cow ghee, cetyl alcohol, and propylene glycol were heated together until melted. Cow urine

Table 1. Composition of cow urine based formulations

Formulation-I	Formulation-II	Formulation-III	Formulation-IV
Poly vinyl alcohol -8, 10, 12g (Ia, Ib, Ic)	Cow ghee-25, 40, 55g (IIa, IIb,IIc)	Cow ghee-25, 40, 55g (IIIa, IIIb, IIIc)	Cow urine double distillate- 25, 40, 55ml (IVa, IVb, IVc)
Gelatin-2 g	Cetyl alcohol-3g	Propylene glycol-6g	SDS -2g
Cow urine double distillate-100 ml	Propylene glycol-12g	Cetyl alcohol-2g	Cetyl alcohol-2g
		SDS-2g	Gelatin-2g
	Freeze dried cow urine- 3g	Gelatin-2g	Mineral oil - to make up to 100ml
	White petroleum jelly-to make up to 100g	Cow urine double distillate-63, 48, 33ml (to make up to 100g)	

N.L.: non lactation, E.L.: early lactation, M.L.: mid lactation, L.L.: late lactation

Table 2. Mean values (mean ± SEM) of various minerals (mg/L) in urine of Deoni cows in non, early, mid and late lactation stages

Mineral/Lactation stage	Calcium	Magnesium	Manganese	Copper	Zinc	Iron
N.L.	0.02933±0.002	0.03533±0.004	0.85733±0.062	0.00367±0.002	0.16267±0.004	0.14267±0.005
E.L.	0.02633±0.005	0.042±0.003	0.79333±0.019	0.00433±0.001	0.15367±0.004	0.12933±0.002
M.L.	0.03133±0.002	0.04633±0.006	0.84167±0.001	0.00267±0.001	0.18167±0.005	0.11967±0.003
L.L.	0.022±0.003	0.02233±0.003	0.91533±0.011	0.006±0.001	0.14133±0.004	0.12667±0.004
Overall	0.02725±0.004	0.03650±0.010	0.85192±0.053	0.00417±0.002	0.15983±0.015	0.12958±0.009

Table 3. Distribution of peaks and probable associated functional groups in Deoni cow urine samples in their non, early, mid and late lactation stages depicted by Fourier Transform Infrared Spectroscopy

Peak	Lactation stage				Associated functional group
	Non	Early	Mid	Late	
430-500	Several(+)	Several (+)	Several (+++)	Several (++)	Aryl disufide (S-S)
500-600	x	580	500, 580	500, 580	Strong C-I Stretching (alkyl halide), Alkyne C-H band
600-700	620, 670	620, 670	620	620	Disuphide linkage (Thiols), C-Br Stretching (alkyl halide)
670-900	700, 770, 820, 850		700, 780, 870	700, 750, 780	700, 750, 780 Disulfides
700 - 800	770	780	750, 780	750, 780	C-Cl Stretching (alkyl halide)
750 - 810 and 860 - 900	770	780, 870	780, 800	780, 800, 850	C-H 1,3 Disubstitution (meta)
800 - 860	820, 850	x	x	850	C-H 1,4-Disubstitution (para) Carbonates, (An Estimation of carbohydrates and glycogen C-OH, C-O, C-O-OC)
950 - 1225	1110, 1200	1020, 1050, 1100, 1180	1030, 1100, 1180	1030, 1100, 1180	Silicate ions, Phosphate, Sulphate, Aliphatic phosphates, C-OH Protein (Serine/threonine, tyrosine (residues of cellular protein)
1000 - 1150	1030, 1110	1020, 1050	1030	1030	C-F Stretching (alkyl halide),
1250 + 800 -890	x	1260, 870	1260, 800	1260, 800, 850	Epoxy and oxirane rings , Peroxides (used in disinfectants)
	1300	1300	1300	1300	Primary and secondary -OH
1450-1510	1465	1465	1465	1465	Carbonate/Carboxylic acid salts (precipitates of proteins)
1590-1690	1590-1700	1590-1700	1590-1700	1590-1700	Open-Chain Imino (-C=N-) group
1700-2000	Several	x	x	x	Simple aromatic compounds
3200-3600	3200-3600	3200-3600	3200-3600	3200-3600	Hydroxyl groups (Broad)

Table 4. Physico-chemical properties of formulation I and II

Parameter	Formulation					
	I a	I b	I c	II a	II b	II c
pH	7.5	7.0	7.8	7.5	7.5	7.3
Firmness (g)	146.5	153.2	167	2076.3	1707.9	1378.3
Adhesiveness (g)	-27.8	-29g	-31.6	-35.9	-31.5	-36.1
Adhesion (g sec)	-13.4	-16.6	-17.2	-31.69	-30.02	-28.94
Consistency (g sec)	358.81	405.5	457.9	2800.91	2852.35	2895.3
Homogeneity	Good	Good	Good	Good	Good	Good
Storage (4º C)	Contamination was observed within 10 days			No bacterial contamination was observed for 6 months		

Table 5. Physico-chemical properties of formulation III and IV

Parameter	Formulation					
	III a	III b	III c	IV a	IV b	IV c
pH	7.5	7.0	7.2	7.5	7.7	7.6
Firmness (g)	36.5	33.2	32.86	48.5	56.2	65.86
Adhesiveness (g)	-7.8	-6	-6.4	-8.8	-9.6	-10.6
Adhesion (g sec)	-4.45	-2.31	-2.35	-8.75	-8.31	-8.35
Consistency(g sec)	68.34	65.5	64.23	258.25	265.8	269.34
Homogeneity	Good	Good	Good	Good	Good	Good
Storage (4º C)	No bacterial contamination was observed for 6 months					

double distillate, gelatin, and SDS were then added. This mixture was stirred with a high-shear mixer at 10,000 rpm for 5 minutes, followed by refrigeration at 4°C for 12 hours.

Formulation IV: Mineral oil and cetyl alcohol were melted together by heating. SDS was dissolved in the cow urine double distillate, and the aqueous phase was gradually combined with the oil phase under continuous high-shear mixing at 10,000 rpm for 2 minutes. Gelatin was then added, and the mixture was stirred again at 10,000 rpm for 10 minutes.

Physico-chemical evaluation of formulations: pH, homogeneity, consistency, firmness, adhesiveness of the formulations was studied using Texture Analyser.

Storage study: The total bacterial count of formulations stored at room temperature was done at monthly interval for six months.

RESULTS AND DISCUSSION

Composition of Deoni Cow Urine

Deoni cow urine was analysed for mineral content across various lactation stages, revealing minerals critical for wound healing processes. Calcium (Ca) levels averaged 0.02725±0.004 mg/L, peaking in the mid-lactation stage (0.03133±0.002 mg/L), while magnesium (Mg) and zinc (Zn) displayed similar trends, with mean values of 0.03650±0.010 mg/L and 0.15983±0.015 mg/L, respectively (table 2). These trace elements, particularly Mg and Zn, are known to support keratinocyte differentiation, cell adhesion and fibroblast activity, facilitating the healing of tissues at the wound site (Zeng *et al.*, 2022; Coger *et al.*, 2019). Additionally, manganese (Mn), with an overall mean of 0.85192±0.053 mg/L and a maximum of 0.91533±0.011 mg/L in the late lactation stage, contributes to cellular proliferation and defence responses in wound healing.

Copper (Cu) and iron (Fe) concentrations, important for collagen synthesis and oxygen transport in healing tissues, were also assessed. Their respective mean values were 0.00417±0.002 mg/L and 0.12958±0.009 mg/L, with Cu peaking in the early lactation stage and Fe in the non-lactation stage. These findings affirm cow urine's potential role in creating a regenerative microenvironment at the injury site, owing to the bioavailability of minerals essential for wound healing.

Fourier Transform Infrared Spectroscopy(FTIR)Analysis

The FTIR analysis of cow urine samples confirm functional groups present in the active principles based on the active peak values in the region of mid-IR spectrum (400-4000 cm-1). This spectrum is divided into four regions (i) single bond region 2500-4000 cm-1), (ii) triple bond region (2000-2500 cm-1), (iii) double bond region (1500-2000 cm-1) and (iv) finger print region (500-1500 cm-1). The schematic IR spectrum of cow urine samples are mentioned in figure 1 and the specific frequency and functional groups in table 3.

FTIR spectrum analysis shows presence of wide

range of compounds with different functional groups. FTIR analysis identified functional groups including alkyl halides, aryl disulfides, silicates, phosphates, and sulfates, along with epoxy and imine groups. Epoxides are particularly noted for their reactivity and pharmacological value, enhancing keratinocyte proliferation and wound re-epithelialization, as reported in recent studies (Powell *et al.*, 2022). Imine groups, found to possess antimicrobial and antifungal properties, support wound healing by providing structural rigidity and enhancing the antibacterial barrier against pathogens (Yang *et al.*, 2016). The presence of these functional groups across lactation stages strengthens cow urine's potential as a wound-healing agent, aligning with findings in other studies on epoxy and imine-containing compounds with significant therapeutic applications (Da Silva *et al.*, 2011).

The antioxidant potential of cow urine and its distillate was evaluated using DPPH radical scavenging assays, revealing notable antioxidant activity across different lactation stages. The double distillate of cow urine exhibited particularly high activity in the late lactation stage, achieving a 43.7% inhibition rate depicted in figure 2. Antioxidants are known to reduce oxidative stress at wound sites, aiding tissue repair by minimizing cellular damage (Nautiyal and Dubey, 2020). These findings highlight cow urine's efficacy as a natural agent that can help to stabilize wound environment and promote healing.

Formulation Development and Stability

Formulations were developed using cow urine distillate combined with mineral oil, cow ghee, and other surfactants to maintain consistency and stability without preservatives as per the contents given in table 1. Sterility tests also indicated that formulation I was prone to contamination within 10 days, while Formulations II, III, and IV remained uncontaminated for six months under refrigeration (figure 3). The consistency of Formulations III and IV was favourable for topical application (table 4, 5) indicating their suitability for potential wound healing applications. Additionally, these formulations are synthesized without preservatives, enhancing their safety profile for use. Further in vivo testing trials will be required to confirm their efficacy.

CONCLUSION

Overall, the mineral composition, active functional groups identified through FTIR, antioxidant properties, and formulation stability underscore the potential of cow urine-based formulations as wound healing agents. The presence of critical minerals and bioactive functional groups aligns with known wound-healing mechanisms, providing a solid basis for future studies and potential wound care applications.

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