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Development and optimization of novel cow urine based formulation and its physiochemical and morphological evaluation studies *in vitro*

Sivaiah Budala[1], Mamta Chauhan[1]*, Manimaran A[1], Dayal Das[1], and Veda murthy G V[1]

Articalinfo

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***Authors:** Dr Mamta Chauhan,
Email: mamtachauhan999@gmail.com, mamta.chauhan@icar.gov.in
ORCID: 0000-0003-4024-0116
1 Southern Regional Station, ICAR-National Dairy Research Institute, Bengaluru-560 030, India

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Abstract

Animal-based formulations have played a vital role in traditional and modern medicine due to their rich composition of bioactive compounds with therapeutic potential. Thus, the study of animal-based formulations bridges traditional knowledge and modern science, offering promising avenues for the development of natural therapeutics with multifaceted health benefits. Hence, the goal of the current study was done to prepare and optimize the cow urine based formulation using cow urine concentrate (CUC) (3% w/w) animal-based components and to evaluate the physicochemical and morphological studies for the first time *in vitro*. The physicochemical properties such as- texture profile, pH, extrudability and rheology study and morphological properties using scanning electron microscopy were evaluated. The results of formulation exhibited a whitish-brown colour, smooth texture, and good extrudability, with a pH of 6.0. These properties make it suitable for topical application. SEM analysis revealed a smooth and homogeneous surface morphology, indicating good stability and uniformity of the formulation. The formulation physicochemical properties after one month of storage at different temperatures, suggesting good shelf-life stability. Hence, in the current study it is concluded that; the cow urine concentrate formulation was successfully optimized and developed *in vitro* that met the relevant pharmaceutical characteristics - safety, potency, stability and shelf life.

Introduction

Animal based formulations have played a vital role in traditional and modern medicine due to their rich composition of bioactive compounds with therapeutic potential. Across various traditional systems of medicine, such as Ayurveda, Unani, and Traditional Chinese Medicine, products derived from animals and their secretions-such as milk, honey, cow urine, ghee, and fish oil-have been utilized for promoting health, enhancing immunity, and treating a wide range of ailments in both humans and animals. These natural animal-derived substances often contain peptides, enzymes, fatty acids, and minerals that exhibit antimicrobial, anti-inflammatory and antioxidant and wound healing properties. The scientific evaluation of such formulations has gained importance in recent years to validate their efficacy and elucidate their mechanisms of action through biochemical and molecular studies. In veterinary and biochemical research, animal-based formulations are increasingly explored as safer and eco-friendly alternatives to synthetic drugs. Their integration with herbal components has shown synergistic effects, leading to development of novel bioactive preparations with improved therapeutic potential. Such combinations are particularly valuable in addressing issues like antibiotic resistance, chronic inflammation, and tissue regeneration. Thus, the study of animal-based formulations bridges traditional knowledge and modern science, offering promising avenues for the development of natural therapeutics with multifaceted health benefits.

A number of topical dermatologic products, ranging from solids to liquids, are available for the treatment of skin diseases (Rawlings and Harding, 2004; Marty, 2002). Topical delivery is attractive as it allows to bring the active components directly to a localized region of the body without exposing the whole organism systemically, thus reducing issues of toxicity and side effects (Walters and Roberts, 2007). Topical delivery is the application of a drug-containing formulation to the skin with the aim of limiting the drug's pharmacological or other effects to the skin's surface or deeper layers in order to treat skin problems. The formulation components and composition, as well as the physicochemical and morphological arrangement of matter in the dosage form can be critical determinants of product performance, because these product quality attributes can modulate the rate and extent to which the API becomes available at the site (s) of action in the skin. A topical formulation must interact with the skin environment after application in order to achieve optimal skin absorption, this interaction may impact the rate at which the component is released and showcased its intense action (Singh *et al.,* 2016). Most topically applied dosage forms are semi-solid. Regarded as a physical body, they combine the properties of liquids and solids. At rest, they behave more like a solid, which, for example, allows a prolonged residence time at the site of application and stability against sedimentation. Under shear, they liquefy and can be spread gently and evenly on the skin surface (Scherphof and Fahr, 2018). The most often and widely used semisolid formulations for topical medication administration are ointments, gels and creams (Verma

et al., 2013). Semisolids constitute a significant proportion of pharmaceutical dosage forms. Topical semisolid products are designed to deliver an active pharmaceutical ingredient (API) to a local site of action in the skin or underlying tissues for the treatment of dermatological, musculoskeletal and other conditions (Roberts *et al.,* 2021; Mohammed *et al.,* 2022).

A semisolid dosage form is advantageous in terms of its easy application, rapid formulation, and ability to topically deliver a wide variety of active drug molecules. Among different topical preparations, ointments are semisolid homogenous formulations prepared for external application to the skin or mucous membranes. Ointments are used topically for several purposes such as protective, antiseptic, emollient, antipruritic, keratolytic, and astringent. Delivery of the drug through the topical route has long been proved to be a promising approach due to the large surface area of the skin, ease of access, wide exposure to the circulatory and lymphatic system, and confined nature of the treatment (Rajalakshmi *et al.,* 2010). Majority of ointments consist of a base, which mainly acts as a carrier or vehicle for the medicaments. The nature of the base also controls its performance; hence, selection of ointment base is a very important aspect of formulation (Cravello and Ferri, 2008). The base of an ointment is of prime importance if the finished product is expected to function. The ointment base composition determines not only the extent of penetration but also controls the transfer of medicaments from the base to the body tissues (Cancalon, 1971).

Pharmacological role of active ingredients used in the components

of the cow urine concentration formulation

The main active ingredients of prepared and optimized semi-solid cow urine concentrate formulation are as follows:

Cow urine from indigenous animals has shown excellent antioxidant, antiseptic, antimicrobial, anti-inflammatory and wound healing properties. Cow urine concentrate formulation on rats showed excellent wound healing activity (Wate *et al.* 2011). The filtered cow urine from *Deoni* breed was subjected to distillation to obtain cow urine distillate, the residue left after the reduction of volume to 1/10th by distillation procedure was designated as cow urine concentrate. But no study has been found till date on *Deoni* cow urine concentrate formulation effectiveness on treatment ailments. Hence, we selected cow urine concentrate for the first time in the current study to prepare and optimize and evaluate its physicochemical and morphological studies *in vitro*. Similarly, White Vaseline is an oily base can able to maintain skin moisture and it creates a highly occlusive, semi-permeable barrier that allows for exchange of water and oxygen and supporting the absorption of active ingredient to the target tissue. Hence, PJ acts as a “wonder jelly”. Smack and colleagues also concluded that “white petrolatum is an effective, safe wound care ointment and is highly cost-effective, efficacious in the treatment of barrier impairment and wound healing compared to antibiotic ointments. Petrolatum is as an active ingredient in the topical formulation preparation and has been shown to be safe and effective for topical use. Petrolatum was found to be comparable and showed superior efficacy than other ointments present in the markets. Its

long history of use and extensive testing have demonstrated that it is non-irritating, is hypoallergenic, and has no systemic toxicity. Hence, in the current study, we selected and used white petroleum jelly as ointment base for preparation of formulation.

Objectives of the current study:

The objective of the current research work is; to formulate the semi-solid ointment using the cow urine concentrate from *Deoni* breed (3% w/w) and to evaluate of physicochemical and morphological evaluation studies for the first time *in vitro*.

Materials and Methods

Materials

Deoni cow urine samples were collected from the cattle yard of the Southern Regional Station, National Dairy Research Institute (SRS-NDRI), Bengaluru, India. Whatman filter papers were purchased from Amazon Private Limited, Bengaluru. Medi-Test Combi 10^{R} VET strips were procured from MACHEREY-NAGEL, Germany. Additional reagents and materials, including - Vaseline white (petroleum jelly), were procured from Hi-Media Laboratories.

Methods

Collection of cow urine samples from *Deoni* breed

Indigenous *Deoni cows* housed at the Livestock Research Centre of the National Dairy Research Institute in Bengaluru, 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.

Urine samples from ten healthy cows of the *Deoni* breed in their late lactation stage were collected randomly in a clean containers during the early morning hours. Samples were filtered with sterile Whatman filter paper and screened for health parameters using "Medi-Test Combi 10^{R} VET" dip sticks (examination of urine) and pooled in equal volume within 1-2 hours.

Distillation and concentration

Cow urine is first filtered through ordinary filter papers or muslin cloth to remove all the visible extraneous matter. The filtered cow urine is subjected to distillation to obtain cow urine distillate (CUD).

The filtered cow urine was subjected to distillation to obtain cow urine distillate, the residue left after the reduction of volume to $1/10^{th}$ by distillation procedure is treated with 5% HCL to obtain a buff coloured solid crystalline matter. This crystalline solid is designated as cow urine concentrate (CUC).

Phytochemical screening of cow urine samples

The freshly prepared cow urine samples from *Deoni* breed were subjected to qualitative phytochemical screening to test for the presence of the phyto-constituents/active principles such as alkaloids, tannins, saponins, phenolics, flavonoids, and terpenoids following standard procedures to identify the constituents as described by Edeoga (2005), Sofowara (1993) and Harborne (1998).

Preparation of formulation

The prepared formulation was made based on the standard formula as described by Paju *et al.* (2013). For preparing the formulation 3g of cow urine concentrate were mixed in 97g

of white vaseline or Petroleum jelly (melted at 60°C) to achieve a homogeneous mixture.

Physico-chemical evaluation of formulation

The optimised formulation was tested for P^H, Extrudability, Texture profile, Rheology flow research, and morphological evaluation by Scanning Electron Microscopy.

P^H

The P^H of prepared formulation was measured using P^H strips. P^H was checked on 0^{th} day and 30^{th} day to check its stability. Data is represented in triplicates (n=3) and is expressed as Mean ± Standard Error (Mean ± S.E).

Extrudability

About 20g of formulation was filled in a clean, aluminium collapsible tube which was sealed at one end and a certain amount of force was applied to the back of the tube in the form of weight and the cap was removed and the ointment was extruded. The percentage of ointment extruded from each collapsible tube on application of a certain amount of force which was evaluated for the samples of formulation stored at 4ºC and Room temperature (26±2ºC) on 0^{th} and 30^{th} day. The formulation extrudability data is represented in triplicates (n=3) and is expressed in (Mean ± S.E) were tested. Each ointment extruded was collected and weighed (g) separately vs. time (sec) taken and the percentage of extruded was calculated and grades were allotted as per the protocol (10 is considered as 100%). The extrudability of prepared formulation was calculated using the following formula:

% Extrudability = [Amount of ointment extruded from the tube/Total amount of ointment filled in the tube] × 100

(> 9 (grade) Extrudability: Excellent)

(> 8 (grade) Extrudability: Good)

(> 6.5 (grade) Extrudability: Fair) (Muthukumar *et al.,* 2021)

Texture profile

Texture profile analysis was performed using TA-XT plus Texture Analyzer and the computer linked to the Analyzer (Stable microsystems, UK). The assessment of consistency/spreadability, adhesiveness, firmness and adhesive force of the formulation was performed using Texture analyzer, while the probe was set to penetrate into the sample containers to a depth of 15 mm at a rate of 2.0 mm/s.

The force exerted to the probe with respect to time was recorded using the Exponent Lite Graph 3.0 Software programme. The measurements were performed while the cone analytical probe was forced down into each sample at 2.0 mm/s test speed to 15 mm depth. The formulation were analysed in triplicates (n=3), the average values and the standard error (Mean ± S.E) were calculated (Mirela Moldovan *et al.,* 2016).

Rheology flow behaviour property

The rheological flow behaviour of the formulation were evaluated using Modular Compact Rheometer (Model: MCR-92, Anton Paar, Austria) equipped with Rheo-Compass software (depicted in Fig. 4) with the help of a 50 mm diameter parallel plate geometry rotor (PP50 probe). The geometric gap between the plates was then adjusted to 1.0 mm. The formulation is allowed to equilibrate at room temperature before the rheological testing.

Approximately 3g of formulation was placed at the centre of the stationary plate and the excess amount of sample protruded beyond the geometry area was carefully removed with the help of a

spatula and the experiment performed. The test temperature for all the measurements was controlled to 25°C to represent the skin temperature during application conditions. The samples were kept at rest for 2 min before shearing and all experiments were carried out in duplicates (n=2). The rheological data for each sample value were presented as Mean ± S.E (Damodar *et al.,* 2018).

Stability study

ICH guidelines were followed for stability study. The formulated formulation was filled in collapsible tubes and stored at different temperatures (Room temperature (26±2°C) and at 4°C), for a period of 1 month and studied for Appearance, pH, and Spreadability, Texture profile and 70 days for Rheology flow study (ICH guidelines, 1993; Dixit *et al.,* 2013).

Morphological evaluation of formulation

The surface morphology, semi-solid particles (Emulsion droplets – Oil-in-water type) size, shape of the prepared and optimized cow urine concentrate formulation was examined in scanning electron microscopy analysis (SEM - JEOL JSMIT 300 Model) at a magnification of 100X.

Statistical analysis

The experiments were performed, and the results were analysed and expressed in Mean ± Standard Error (Mean ± S.E). The statistical test, One Way ANOVA was performed and the test of significance probability (P-value) was tested.

Results and Discussion

Preparation of cow urine concentrate

Urine samples from ten healthy cows of the *Deoni* breed in their late lactation stage were collected randomly in a clean containers and were filtered with sterile Whatman filter paper. The filtered cow urine was subjected to concentration process. Cow urine left after the reduction of volume to 1/10th by distillation procedure was designated as cow urine concentrate (Wate *et al.,* 2011) is depicted in Fig. 1.

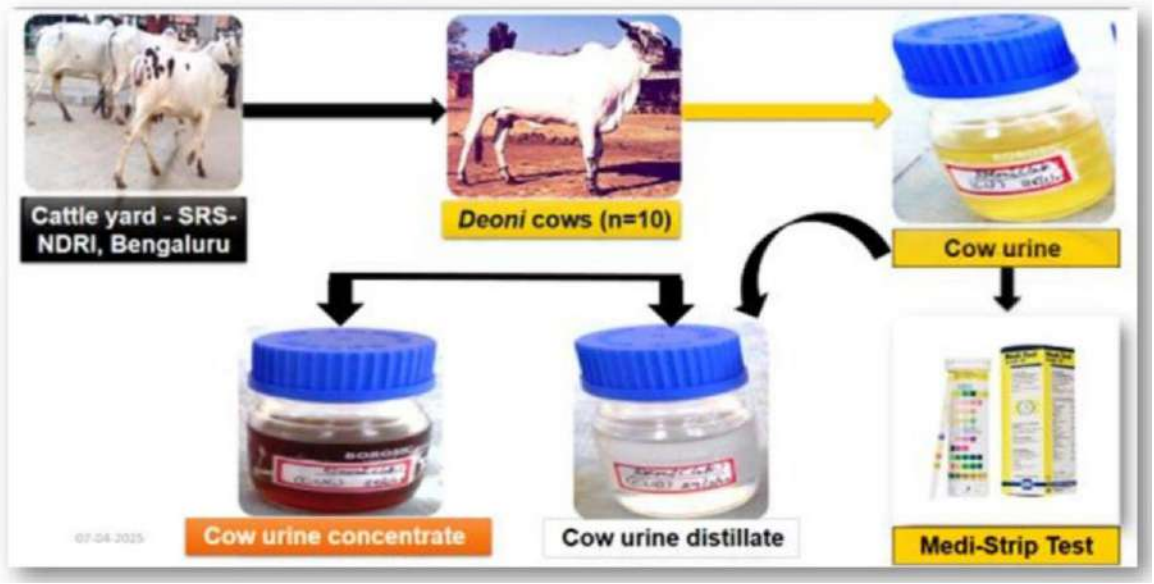


Fig. 1 Collection of *Deoni* cow urine samples and its cow urine distillate and concentrate preparation

Active principles

Qualitative phytochemical analysis was conducted to confirm the presence of active principles in cow urine concentrate exhibited a broader range of phytochemicals, including phenols, alkaloids, flavonoids, saponins, terpenoids, and tannins. These findings are summarized in Table 1.

Composition of formulation

The prepared and optimized cow urine concentrate formulation is depicted in Fig. 2. The composition of formulation was described in Table 2.

Fig. 2 Picture of optimized *Cow urine concentrate* formulation

Properties of formulation

The formulation was prepared and stored at 4ºC and RT (26 ± 2ºC). The formulation had whitish brown colour, semi-solid homogeneous state, smooth texture, good extrudability with pH 6.0. The storage of formulation physico-chemical properties at different temperatures for 30 days were not changed. Physico-chemical characteristics were observed on day 0 and 30 was mentioned in Table 3.

Texture profile analysis

Texture profile analysis

Texture profile of formulation analysis using texture analyser is depicted in Fig. 3a and their stability graphs are shown in Fig.3b and the texture profile analysis of *cow urine concentrate* formulation at 4ºC and RT (26 ± 2ºC) storage temperatures for 30 days were mentioned in Fig. 3c.

Fig. 3a Texture profile analysis of *Cow urine concentrate* formulation using Spreadability Rig (HDR/SR)

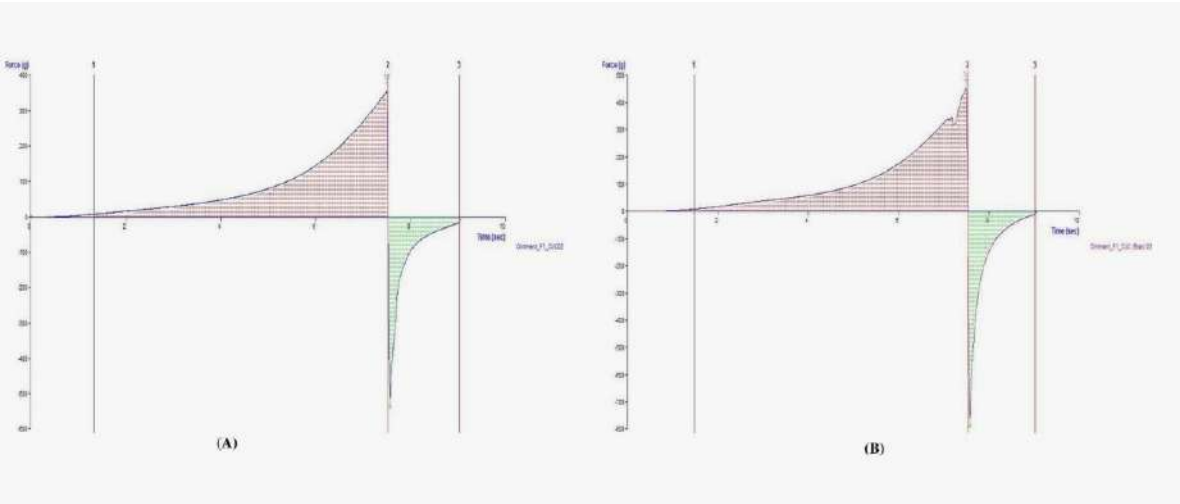
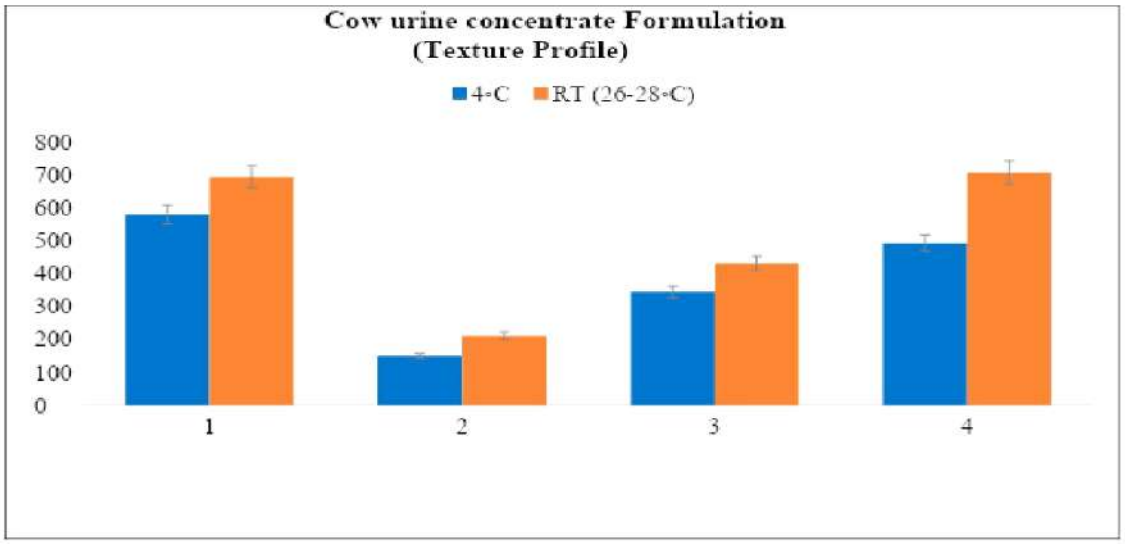


Fig. 3b Texture profile stability graphs of *cow urine concentrate* formulation at (A) 0th day and (B) 30 days



Values are represented in triplicates (n=3) (Mean ± S.E.M)

Statistically significant difference within and between the storage temperatures texture profiles (P<0.05)

Fig. 3c Texture profile analysis of *cow urine concentrate* formulation at 4ºC and RT (26 ± 2ºC) storage temperatures for 30 days

Consistency/Spreadability

The formulation has less consistency or high spreadability on 0th day (579.5 ± 29.2) while its reverse is observed on day 30 (693.6 ± 20.8).

Adhesiveness

On the 0th 30th day, the adhesiveness of the formulation was (-148.9 ± 8.5 and -210.8 ± 5.9) respectively. Adhesiveness was observed more on 30th day.

Firmness

The firmness of the formulation on the 0th and 30th day was (342.6 ± 14.1) and 431.2 ± 22.1) respectively indicating that the firmness of the formulation was higher on the 30th day. In other words, it was less spreadable on the 30th day than 0th day.

Adhesive force

On the 0th and 30th day, the adhesive force for the formulation was (-492.5 ± 23.7 and -706.9 ± 57.2) respectively. On the 0th day, the adhesive force of the formulation was less increased after 30 days. The ointment formulation was stickier

on the 30th day than observed on 0th day.

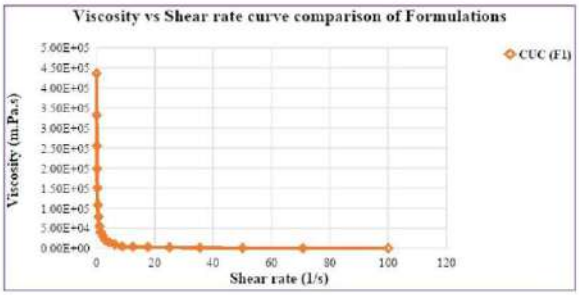
Rheological flow behavior characteristics

The rheological flow behaviour of formulation such as shear rate vs. viscosity and shear rate vs. shear stress at 4ºC and RT (26 ± 2ºC) storage temperatures for 70 days' were evaluated with the help of Modular Compact Rheometer (Anton Paar, Model - MCR-92, Austria) is depicted in Fig. 4a.

The Shear rate Vs. Viscosity measurement of formulation at 4ºC and RT (26 ± 2ºC) storage temperatures for 70 days

The shear rate vs. the viscosity of the formulation causing a spreading action as the shear rate increases from (0.997 to 100 Sec^{-1}), in a step-wise manner and the viscosity was decreases gradually from (4, 36,000 – 348.5 m.Pa.s). Similarly, the shear rate vs. the viscosity of a formulation as the shear rate increases from (0.997 to 100 Sec^{-1}), and the viscosity decreases gradually from (6, 04,000 – 3100 m.Pa.s) and which is explained in viscosity flow curve. The shear rate vs. the viscosity measurement of the formulation at 4ºC and RT (26 ± 2ºC) storage temperatures for 70 days was depicted in Fig. 4b.

Fig. 4a Rheology flow behaviour analysis of *Cow urine concentrate* formulation using MCR 92 Rheometer



Values are represented in duplicates (n=2) (Mean ± S.E.M)

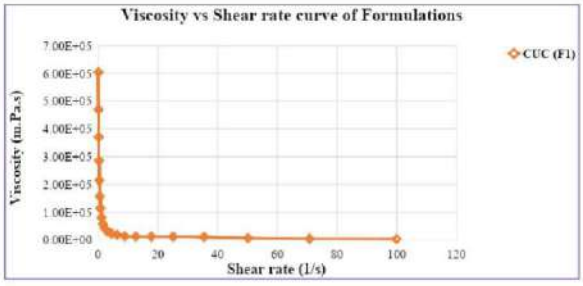


Fig. 4b Cow urine concentrate Formulation shear rate vs viscosity flow curve at 4ºC and RT (26 ± 2ºC) storage temperatures for 70 days

The Shear rate Vs. Shear stress measurement of formulation at 4ºC and RT (26 ± 2ºC) storage temperatures for 70 days.

Shear rate is plotted on the x-axis, shear stress on the y-axis. As the shear rate increases from (0.997 to 100 Sec^{-1}) in a step-wise manner, the shear stress decreases initially and then increases and again decreases (randomly) from (68.8 – 32.6 Pa). Similarly, the shear rate is plotted on the x-axis, shear stress on the y-axis. As the shear rate increases from (0.997 to 100 Sec^{-1}) in a step-wise manner, the shear stress decreases randomly from (60.2 – 300.8 Pa). The Shear rate Vs. Shear stress of the formulation at 4ºC and RT (26 ± 2ºC) storage temperatures for 70 days was depicted in Fig. 4c.

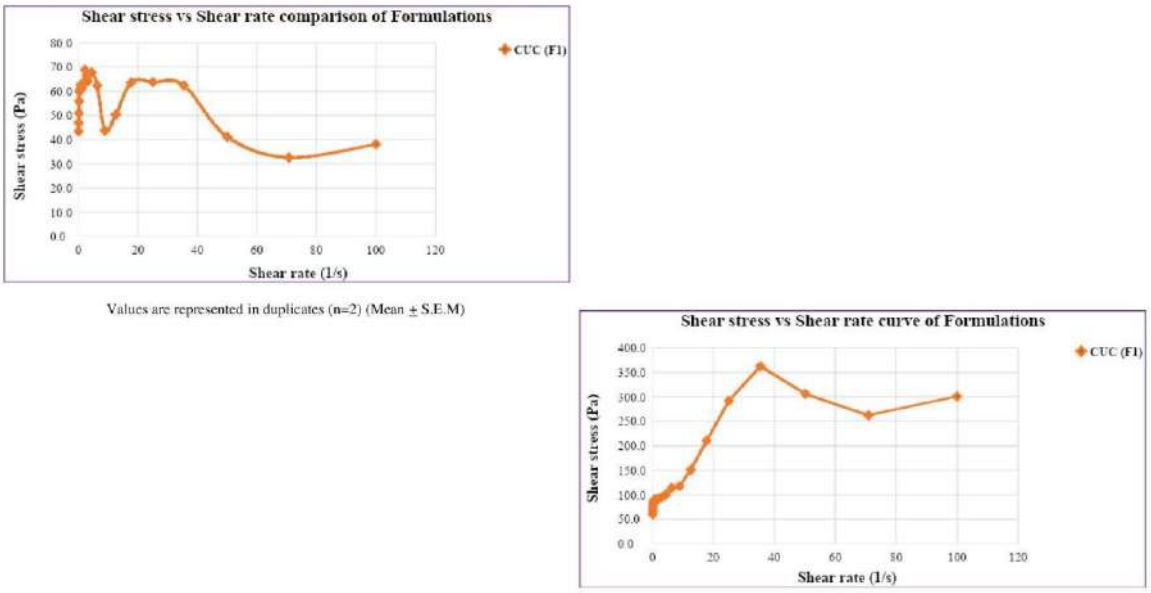


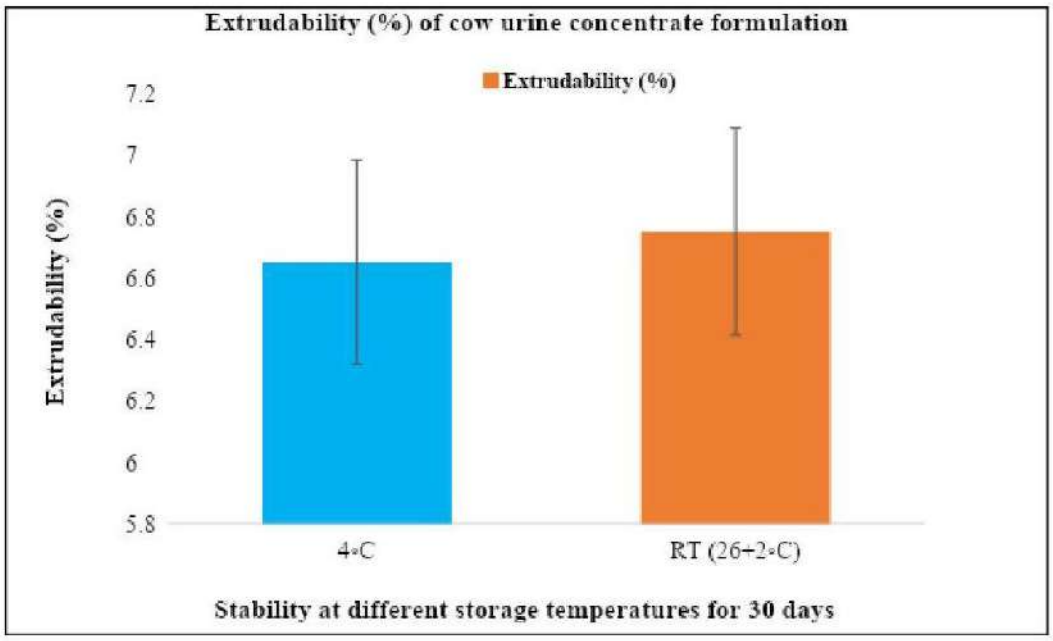
Fig. 4c Cow urine concentrate Formulation shear rate vs shear stress flow curve at 4ºC and RT (26 ± 2ºC) storage temperatures for 70 days

Extrudability

The extrudability of the formulation at 4ºC and RT (26 ± 2ºC) storage temperatures was depicted in Fig. 5. At 4ºC the extrudability was 6.65% and at RT (26 ± 2ºC) the extrudability was 6.75% respectively.

Morphological evaluation of formulation

Scanning electron microscopy analysis showed that the vesicles are irregular in shape and size and the external morphology was smooth in appearance Fig. 6.



Values are represented in triplicates (n=3) (Mean ± S.E.M)

Statistically significant difference within and between the storage temperatures texture profiles (P<0.05)

Fig. 5 Extrudability of cow urine concentrate formulation at 4ºC and RT (26 ± 2ºC) storage temperatures for 30 days

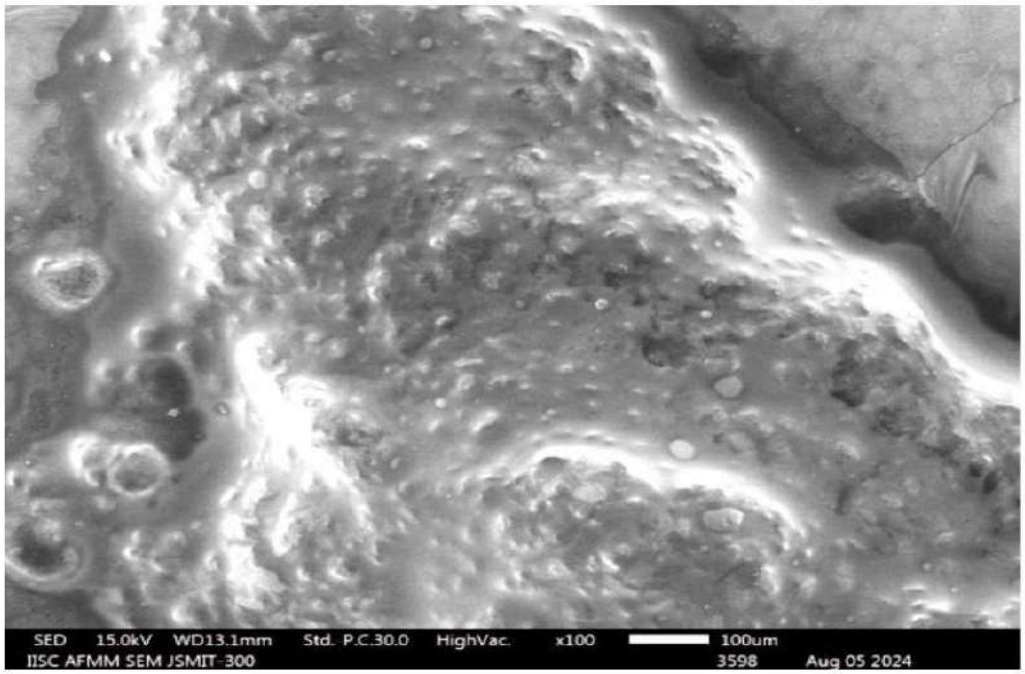


Fig. 6 Morphological evaluation of *Cow urine concentrate* formulation by scanning electron microscopy

In the current study was done to prepare the cow urine based formulation using cow urine concentrate (3% w/w) animal-based components and to evaluate the physicochemical and morphological studies for the first time *in vitro*.

Urine samples from ten healthy cows of the *Deoni* breed in their late lactation stage were collected randomly in a clean containers and were filtered with sterile Whatman filter paper and the filtered cow urine was subjected to distillation, the residue left after the reduction of volume to 1/10th by distillation procedure was designated as 'Cow urine concentrate' (Wate *et al.,* 2011).

Raw cow urine is highly dilute and complex biological fluid is mostly water (about 95-98%), with only 2-5% solids which include urea, creatinine, phenols, volatile fatty acids, enzymes, various minerals and with small amounts of organic and

inorganic solutes are present in trace amounts. Early morning first voided cow urine is more sterile and has more macro and micronutrients along with other enzyme/urea content could be more effective. The main of cow urine is phenols. Phenols are bactericidal to *gram positive* and *gram negative* bacteria. Therefore, the presence of phenols in cow urine has a potent anti-microbial activity. The fresh cow urine contains more amount of phenol and hence has a better anti-microbial activity (Jarald *et al*., 2008). Fresh cow urine decomposes quickly due to microbial growth and enzymatic reactions (like urease breaking down Urea to Ammonia and CO_2). Cow urine has different fractions like anti-microbial activity due to the presence of certain components like volatile and non-volatile ones (Jarald *et al.,* 2008). Presence of creatinine, urea, swarnakshar (aurum hydroxide), phenols, carbolic acid, calcium and manganese has strongly explained the germicidal and anti-microbial properties of cow urine. Presence of amino acids and urinary peptides may enhance the bactericidal effect by increasing the bacterial cell surface hydrophobicity. Fresh cow urine contains higher amounts of phenols makes it more effective against microbes. After photo-activation, few biogenic volatile inorganic and organic compounds such as; CO_2, NH_3, CH_4, methanol, propanol, acetone and some metabolic secondary nitrogenous products are also formed (Upadhyay *et al.,* 2010). Cow urine contains phenolic acids (gallic, caffeic, ferulic, o-coumaric, cinnamic and salicylic acids) which have anti-fungal characteristics (Singh *et al.,* 2012). When we want to analyse active compounds, direct analysis of raw urine is not effective. Hence, distillation and concentration are

essential preparatory steps for separating organic compounds of interest.

Distillation helps separate volatile (phenols, aldehydes, short chain acids) and non-volatile substances, removing unwanted interfering substances before testing for analysis. Distillation and concentration of urine samples are crucial to remove water and impurities, enrich the organic constituents, and improve the accuracy and sensitivity of bioactive compound analysis. Distillation (evaporation) removes the bulk water, leaving behind a concentrated residue containing the. This concentration step improves the detection sensitivity of various analysis. Without concentration, the extraction efficiency is poor. After concentration, FTIR or NMR signals become stronger and clearer, making it possible to detect specific active compounds.

By concentrating urine (through evaporation or distillation; the bioactive compounds (urea, uric acid, volatile fatty acids, phenols and hippuric acid) become more potent per unit volume. This improves the biological activity and therapeutic efficacy in formulations. The concentrated urine reduces the volume needed for storage and transport and allows easier incorporation into the formulations. Concentration helps to standardize the active fractions and allow for quantitative estimation of key biomarkers for batch-batch consistency. Concentrated urine acts as a bio-enhancer (increasing absorption or activity of metabolites). Concentration-especially after mild heating or distillation –inactivates enzymes and reduces microbial load, adjusting P^H improves stability. Concentration

removes volatile ammonia and offensive odour (partially), microbial contaminants and organic debris. Thus making it more acceptable and safer for pharmaceutical or veterinary formulations.

Qualitative phytochemical analysis was conducted to confirm the presence of active principles in cow urine concentrate exhibited a broader range of phytochemicals, including phenols, alkaloids, flavonoids, saponins, terpenoids, and tannins. These findings are summarized in Table 1. These phyto-constituents are known for their significant antioxidant, antibacterial, and anti-inflammatory properties, indicating the potential therapeutic efficacy strongly supports in the cow urine based novel formulation in wound healing applications.

In the present study aimed to evaluate the semisolid cow urine concentrate formulation i.e., ointment were prepared by using active ingredients (Table 2). The formulation was prepared and packed in a pharmaceutical aluminium collapsible tube. The packaging plays a role in maintaining the integrity, safety, stability and efficacy of medications. These tubes offer a unique combination of protective properties, regulatory compliance, and used convenience, making them ideal solution for sensitive pharmaceutical formulations. These tubes can also provide high level of protection of pharmaceutical products, especially those containing active ingredients that are sensitive to environmental factors.

The formulation was stored at 4ºC and RT (26 ± 2ºC). A refrigerator set to 4ºC is considered safe for formulations storage. The 4ºC is the logical minimum for a refrigerator because below that ice crystals start forming which many time destroy the texture and quality of the

formulation. The 4ºC storage temperature the more inactive the decay bacteria and molds. Hence, the stability and quality of formulations properties was not changed. The standard room temperature for a laboratory is typically between 20-28 ºC. High temperatures can accelerate the degradation of active ingredients and excipients present in the formulation, leading to reduced efficacy and shelf life. This might be reason behind the slightly increase in physicochemical parameters at RT (26 ± 2ºC) than at 4ºC storage temperatures *in vitro*.

In the current study, the formulation was prepared and stored at 4ºC and RT (26 ± 2ºC) storage temperatures. The formulation had whitish brown colour, semi-solid, homogeneous state, smooth texture, good extrudability with pH 6.0. The storage of formulation physico-chemical characteristics were observed on day 0 and 30 was mentioned in Table 3. Portela *et al*. (2019) emphasized the importance of physico-chemical properties in the development of effective wound-healing formulations. SEM analysis revealed a smooth and homogeneous surface morphology, indicating good stability and uniformity of the formulation. The formulation suggesting good shelf-life stability. This might be due to the formulation was found to be in the acceptable range. Negut *et al*. (2020) highlighted the role of stable formulations in ensuring consistent therapeutic efficacy.

The P^H of semi-solid ointment formulation is crucial factor that can significantly impact the effectiveness, safety, stability and shelf life on the skin of experimental animals. P^H is the most important and versatile parameter in terms of safety, stability and shelf life of

ointment formulations. P^H is a potential critical quality attribute of topical formulations. P^H may affect drug stability and physicochemical properties of semi-solid products like emulsion stability and rheological properties. The P^H test aimed to ensure the safety of the ointment formulations preparations by verifying that they would not cause skin irritation upon topical application. The P^H should be compatible with the skin's natural P^H (4.5-5.5) or (5.0-6.5) which is good for skin pH to avoid irritation and to ensure optimal absorption. This might be the reason behind the stability and quality of formulations without change in pH (6.0) which was observed in our study. The P^H of an ointment can affect the chemical stability of its active ingredients. Some ingredients are stable only within a specific P^H range and can degrade, if the P^H shifts outside this range. Skin membrane acts as a P^H dependant permeable-selective barrier to permeation of drug molecules. It was reported that drug permeability through skin barrier membrane is a function of P^H of formulation vehicle applied. Hence, P^H appears to be one of the most critical quality attribute of topical formulation.

Texture profile analysis (TPA) is the most popular method of texture analysis and to evaluate the semi-solid ointment formulations with better efficacy now a day. Ointment formulation is one of the key properties to evaluate when determining the quality, safety and acceptability and embraces a large number of textural characteristics. TPA is a technique that has been widely employed for determining the textural properties of ointment formulation's performance. Textural attributes of the ointment formulations namely; consistency (spreadability), firmness (hardness),

adhesiveness and adhesive force (stickiness) etc. were evaluated with the help of TA-XT plus Texture Analyzer in the Compression mode.

In the current study, the texture profile analysis of the *cow urine concentrate* semi-solid ointment formulation and its textural attributes such as; firmness (hardness), consistency (spreadability), adhesiveness and adhesive force (stickiness) etc. were evaluated with the help of TA-XT plus Texture Analyzer (Stable systems, U.K) with Spreadability fixture with 90° cone probe which can penetrate into the ointment formulation at a distance of 15 mm with 2.0 mm/sec test speed (Fig. 3a) and to determine the formulation maximum positive force on positive peak (firmness); maximum negative force on second peak (adhesive force); area of positive peak (spreadability/consistency) and area of negative peak (adhesiveness) with the help of the Exponent Lite Graph 3.0 software programme to plot the force-time graph (Fig. 3b).

Generally, the consistency is inversely proportional to the spreadability. The formulation has less consistency or high spreadability on 0^{th} day (579.5 $\pm$ 29.2) while its reverse is observed on day 30 (693.6 $\pm$ 20.8). When the consistency of the formulation is increases, the spreadability was decreases and vice-versa. In this context, on 0^{th} day, the ointment formulation consistency was increases but the spreadability was decreases. Similarly, at 30^{th} day, the ointment formulation consistency was decreases but the spreadability was increases. Spreadability is the ease with which a formulation can be applied to a thin, even layer of skin. Good spreadability is crucial for ease of application on wounds of animals with uniform coverage. Therefore, the consistency/spreadability is one

of the most important quality parameters of texture profile analysis.

Adhesiveness is an important attribute of semi-solid ointment formulations. Some formulations exhibit a marked tendency to adhere to a contact surface, which is generally known as Adhesiveness. A cone type probe applies a specified force to the surface of the ointment formulations for a specified time (to achieve a good contact between the two surfaces) and then moves away from the formulation at which point the force to separate the two surfaces is measured and used as a measure of ointment formulation stickiness/adhesiveness/tackiness.

On the 0^{th} 30^{th} day, the adhesiveness of the formulation was (-148.9 $\pm$ 8.5 and -210.8 $\pm$ 5.9) respectively. Adhesiveness was observed more on 30^{th} day and slightly less on 0^{th} day. High adhesiveness might indicate on 30^{th} day leads to better retention of the skin but could also lead to a sticky feeling. Therefore, adhesiveness is one of the most important quality parameters of texture profile analysis.

Firmness or hardness may be measured by the maximum force (g) required to obtain a given deformation or by the amount of deformation under given force. The maximum positive peak value on the graph of extract ointment formulation is a measure of the firmness of the formulation at the specified depth. A higher positive peak load (firmness) and a hardness value of the work performed indicates the formulation is less spreadable and vice-versa. The firmness of the formulation on the 0^{th} and 30^{th} day was (342.6 $\pm$ 14.1) and 431.2 $\pm$ 22.1) respectively indicating that the firmness of the formulation was higher on the 30^{th} day. In other words, it was less

spreadable on the 30th day than 0th day.

If the adhesive force is higher in the formulations, the stickiness also high. The maximum negative peak indicates the stickiness of the formulation and the negative area is taken as the work of adhesion (adhesiveness). A stickier formulation will require a greater force to remove the probe yielding a larger negative area. The maximum negative force on the graph indicates the adhesive strength of the formulation; the more negative part of the value, the more 'sticky' the formulation. On the 0th and 30th day, the adhesive force for the formulation was (- 492.5 ± 23.7 and - 706.9 ± 57.2) respectively. On the 0th day, the adhesive force of the formulation was slightly lower than 30 days. The ointment formulation was stickier on the 0th day than observed on 30th day with better adhesive ability. The overall texture profile analysis of *Acalypha indica* formulation at 4ºC and RT (26 ± 2ºC) storage temperatures for 30 days were mentioned in Fig. 3c. One way ANOVA was performed to test the level of significance (P-value) with in each storage temperature and also between the storage temperatures. There was significant difference within and between the storage temperatures texture profiles ($P<0.05$).

The rheological flow behaviour of formulations such as shear rate vs. viscosity and shear rate vs. shear stress at 4ºC and RT (26 ± 2ºC) storage temperatures for 70 days' were evaluated with the help of MCR-92 Rheometer (Anton Paar, Austria) (Fig. 4a).

The shear rate vs. the viscosity of the formulation causing a spreading action as the constant shear rate increases from (0.997 to 100 Sec^{-1}), in a step-wise manner and the viscosity was decreases gradually

from (4, 36,000 – 348.5 m.Pa.s). Similarly, the shear rate vs. the viscosity of a formulation as the shear rate increases from (0.997 to 100 Sec^{-1}), and the viscosity decreases gradually from (6, 04,000 – 3100 m.Pa.s) and which is explained in viscosity flow curve. This clearly indicates that a breakdown of the internal structure (deformation) of the formulation and the orientation reduces internal resistance of formulation and allows a greater rate of shear, and as a result, decrease in apparent viscosity called 'Shear thinning'. High viscosity at low shear rates is usually desirable behaviour as it extends shelf life and makes the product easier and more accurate to apply to specific skin areas. A low viscosity at higher shear rates, on the other hand, makes it easier for the consumer to spread and rub the product in and allows for faster absorption of formulation active ingredients into/through the skin. The shear rate vs. the viscosity measurement of the formulation at 4ºC and RT (26 ± 2ºC) storage temperatures for 70 days was depicted in Fig. 4b.

Shear rate is plotted on the x-axis, shear stress on the y-axis. As the constant shear rate increases from (0.997 to 100 Sec^{-1}) in a step-wise manner, the shear stress decreases (randomly) from (68.8 – 32.6 Pa). Similarly, the shear rate is plotted on the x-axis, shear stress on the y-axis. As the shear rate increases from (0.997 to 100 Sec^{-1}) in a step-wise manner, the shear stress increases from (60.2 – 300.8 Pa) and this might be due to formulation shear thinning ability while rotating the probe. The Shear rate vs. the Shear stress graph says that shear stress and shear rate are inversely related. When we increase the shear rate, the shear stress decreases randomly. The Shear rate Vs. Shear stress of the formulation at 4ºC and RT (26 ± 2ºC)

storage temperatures for 70 days was depicted in Fig. 4c.

Based on overall rheology data analysis, we observed and confirmed that at 4ºC and RT (26 ± 2ºC) storage temperatures for 70 days, the rheological flow behaviour of formulation was shown dissimilar trend but slight variation at both the storage temperatures without affecting its stability and quality (at (26 ± 2ºC) has better flow behaviour than 4ºC through the graphical observations). This rheological flow behaviour differences at 4ºC and RT (26 ± 2ºC) storage temperatures, it might be due to the ratio of composition of formulation, and excipient (base material used) and it directly impacts the rheological properties of the formulation effectively and subsequently influences its extrusion, extensibility, stability and ultimately quality of formulation.

The extrudability is an important physico-chemical parameter to determine the ease of removal and application of ointment formulations from its packaging. Extrudability can be assessed based on the ability to dispense the ointment formulation smoothly and completely from the tube without excessive force applied. Ointment formulations are considered acceptable; if they demonstrate easy extrudability without any blockage or inconsistent flow from the tube. Hence, the extrudability is one of the pivotal parameter for ointment formulations analysis and to check their effectiveness in ease of application. In our study, the extrudability of formulation evaluated at different storage temperatures, we observed that at 4ºC storage temperature has slightly less extrudability (6.65%) than Room Temperature (26 ± 2ºC) (6.75%) it clearly showcased the slightly better

extrusion from the tube at Room Temperature (26 ± 2ºC) than 4ºC storage temperature (Fig. 6). One way ANOVA was performed to test the level of significance (P-value) with in each storage temperature and also between the storage temperatures. There was significant difference within and between the storage temperatures extrudability profiles ($P<0.05$).

The shelf life of ointment formulations can vary significantly based on their physical state and composition. Semi-solids such as ointment formulations and their shelf life is typically shorter than solids but longer than liquids. They are more susceptible to physical and chemical stability, but advancements in formulation design have improved their stability. A stability study is a designed experiment where the pharmaceutical product is stored in environmental chambers and followed for a prescribed amount of storage time. The most important steps during the developmental stages include pharmaceutical analysis and stability studies that are required to determine and assure the identity, potency and purity of ingredients, as well as those of the formulated products.

Scanning electron microscopy analysis showed that the vesicles are irregular in shape and size and the external morphology was smooth in appearance Fig. 6.

In the current study, overall the *cow urine concentrate* formulation exhibited physicochemical properties such as P^H, Texture profile and Extrudability for a period of one month storage at 4°C and Room Temperature (26 ± 2ºC) temperatures with mild changes, suggesting good shelf-life stability. Similarly, the rheology flow behaviour of the formulation

optimized at two different temperatures at 4°C and Room Temperature (26 ± 2ºC) for a storage period of 70 days were evaluated. We observed that, there was slight changes of rheology profiles at 4°C and (26 ± 2ºC) Temperatures without affecting its stability, consistency and shelf life of the optimized formulation. Hence, it is concluded that, the prepared and optimized formulation was successfully developed that met the relevant pharmaceutical characteristics for topical applications.

Conclusion

In the current study, the cow urine concentrate formulation was prepared and optimized *in vitro*. The results of optimized formulation exhibited a whitish-brown colour, smooth texture, and good extrudability, with a pH of 6.0. These properties make it suitable for topical application. SEM analysis revealed a smooth and homogeneous surface morphology, indicating good stability and uniformity of the formulation. The formulation physicochemical properties after one month of storage at different temperatures, suggesting good shelf-life stability. This might be due to the skin's natural P^H (4.5-5.5) or (5.0-6.5) which is good for skin to avoid irritation and to ensure optimal absorption and showcased good stability and quality of formulations which was observed in the present study acceptable range. From the present study it can be concluded that; the cow urine concentrate formulation was successfully optimized and developed that met the relevant pharmaceutical characteristics *in vitro*.

Declarations

Conflict of interest statement

The author (s) clearly certify that there is no conflict of interest in any manner with regard to the content/research work presented in the manuscript, briefly as an original research article for submission to FAAI Journal for publication.

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Author contribution declaration

Sivaiah Budala: Conceptualization, Methodology, Writing-Original draft, Formal data analysis

Mamta Chauhan: Conceptualization, Project administration, Visualization, Supervision, Resources, Editing.

Manimaran A.: Resources and Editing.

Dayal Das: Methodology and Editing

Vedamurthy G.V.: Methodology and Editing.

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Table 1. Phytochemical screening of cow urine concentrate

Qualitative test	Active principles	Cow urine Concentrate
Wagner's	Alkaloids	+
Ferric Chloride	Tannins	+
Foam test	Saponins	+
Ferric Chloride	Phenolics	+
Shinoda/Lead acetate	Flavonoids	+
Liberman Burchard	Terpenoids	+

(+) indicates presence of phyto-chemicals

Table 2. Composition of cow urine concentrate formulation

Cow urine concentrate formulation (3% w/w)	
Cow urine concentrate	3g
Petroleum Jelly (or) White Vaseline	97g

Table 3. Physico-chemical characteristics of cow urine concentrate formulation

Characteristics	Observations	
	0^{th} day	**30^{th} day (Stability)**
Colour	Whitish Brown	Whitish Brown
Odour	Characteristic	Characteristic
State	Semi-solid	Semi-solid
Washability	Good	Good
Texture	Smooth	Smooth
P^H	6.0 ± 0	6.0 ± 0
Rheology flow behaviour	Good	Good
Extrudability	Ok	Ok
Consistency	Good	Good
Spreadability	Good	Good
Homogeneity	Homogenous, Uniform distribution, Soft & Consistent	Homogenous, Uniform distribution, Soft & Consistent