JOURNAL OF NATURAL REMEDIES
DOI: 10.18311/jnr/2023/31414
RESEARCH ARTICLE

Physicochemical Characterization and Cytocompatibility Study of Lyophilized Cow Urine Powder

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Abstract

Cow urine is known for possessing therapeutic potency which is found to vary depending upon its physical forms such as fresh sterile urine, distillate, lyophilized powder, etc. In the current work, we demonstrate the physicochemical characteristics and cytocompatibility of lyophilized powder of cow urine obtained from *Sahiwal* and the pure Indian breed. We have used several analytical techniques, including Thermogravimetric Analysis (TGA), Fourier Transform Infrared (FTIR) Spectroscopy, Differential Scanning Calorimetry (DSC), Nuclear Magnetic Resonance (NMR) spectroscopy and Scanning Electron Microscopy – Energy Dispersive X-ray Spectroscopy (SEM-EDX) to examine the physical and chemical nature of cow urine powder obtained through lyophilization. SEM-EDX analysis showed clusters like morphology and revealed 25-30% of metal ions present in both samples. TGA analysis showed three steps of degradation sequentially most likely due to initial desorption of adsorbed water, urea degradation, and loss of glycoside moiety present in flavonoid. DSC data revealed the presence of both irregular (amorphous) and ordered molecular structures (crystalline) in the lyophilized powder of cow urine. ^{1}H NMR spectra of all the prepared cow urine samples exhibited a similar trend and did not vary significantly and; confirming the presence of the same flavonoid/compounds in each sample. FTIR spectra of all the prepared cow urine samples exhibited a similar trend and did not vary significantly as observed through ^{1}H NMR spectra. In addition, a cytocompatibility study and MTT assay using NIH-3T3 fibroblast cells revealed that lyophilized cow urine powders possessed no deleterious effects on healthy fibroblast cells. Thus, the outcomes provide a benchmark for further understanding of the lyophilized form of cow urine that could be potentially useful for analyzing its therapeutic value.

Keywords: Analytical Techniques, Fibroblast Cells, Indian Breed, *Sahiwal*

1. Introduction

The cow is a very popular animal since the very beginning of human civilization[1,2]. Its popularity is because of various products obtained from it for the use of mankind. In the Western world especially in the Muslim and Christian communities cow is mainly used for its products like milk and meat as the source of protein[3]. While in the Eastern world, cow is used for its milk, urine, cow dung, curd, and ghee[4]. In a country like India, the Indian breed of cow has got a very special and divine position since ancient times. In the Hindu religion, the cow is considered as a 'mother' as the constituents of human mother's milk and cow's milk are almost similar, and hence both are life-supporting[4].

'*Panchagavya*', a mixture made by mixing direct constituents (milk, urine, and dung) and indirect constituents (ghee and curd) of cow, has got a special therapeutic value since time immemorial[5]. Different Ayurvedic literatures have reported the pharmaceutical

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Article Received on: 08.10.2022 Revised on: 27.04.2023 Accepted on: 08.05.2023

and therapeutical applications of cow urine for the prevention and management of many diseases. Several research works have reported that cow urine is effective in the treatment of heart attack, blood pressure, diabetes, arterial blockage, asthma, arthritis, thyroid, psoriasis, piles, eczema, AIDS, cancer, and several other diseases[6]. Cow urine is known to contain water ranging nearly 95%, urea up to 2.5% and the remaining (i.e., 2.5%) consists of a variety of salts, minerals, enzymes, and hormones[7]. It contains amino acids, cytokines, lactose, uric acid, urea, phosphorus, calcium, iron, potassium, and enzymes, etc[7].

In India, several research works are currently being carried out on cow urine for examining its wide spectrum activity. In agriculture, cow urine is used for improving soil fertility[8]. It has also been used as a biopesticide[9]. Therapeutically cow urine is used in treating various skin diseases like eczema itching, sunburn, etc[2]. Cow urine is also used in stones, stomach, liver, kidney, and heart diseases[10]. Cow urine also possesses significant antidiabetic activity[11,12]. Cow urine also serves as an immunostimulant and possesses appreciating anticancer activity[13,14,17]. Cow urine is known to possess potent wound healing properties[15,16]. It is also considered as an effective antioxidant and antimicrobial agent[14,17]. To utilize cow urine for biomedical applications, its various forms such as distillate, lyophilized form, etc. have been developed and examined for its potential efficacy[14]. In the current work, we demonstrate the physicochemical properties of lyophilized cow urine to establish the characteristics of the powder form of cow urine obtained through lyophilization. We have used several analytical techniques, including Thermogravimetric Analysis (TGA), Fourier Transform Infrared (FTIR) Spectroscopy, Differential Scanning Calorimetry (DSC), Nuclear Magnetic Resonance (NMR) Spectroscopy and Scanning Electron Microscopy – Energy Dispersive X-Ray Spectroscopy (SEM-EDX) to examine the physical and chemical nature of cow urine powder processed through lyophilization. In addition, a cytocompatibility study of cow urine using an MTT assay and NIH-3T3 fibroblast cells was performed.

2. Materials and Methods

2.1 Collection of Cow Urine

Cow urine samples were obtained from the dairy farm of Banaras Hindu University, Varanasi, India which maintains a variety of different breeds of cow. The cow selected for our research work was a healthy *Sahiwal* and a pure Indian breed (*desi*) aged around seven years having a healthy diet uniformly and having undergone a regular vaccination schedule.

The first urine of the cow was obtained fresh early in the morning in screw-capped sterile bottles and brought to the laboratory for further analysis and examinations. Fresh cow urine was subjected to testing after filtration with ordinary filter paper. After that, the filtered cow urine was subjected to lyophilization. The samples were classified as mentioned below:

Sample 1: Cow urine obtained from *Sahiwal* breed

Sample 2: Cow urine obtained from pure Indian breed

2.2 Scanning Electron Microscopy (SEM) Analysis with Energy-Dispersive X-Ray Spectroscopy (EDX) Measurement of Freeze-Dried Cow Urine Powder

The external appearance and particle size were examined using Scanning Electron Microscopy (SEM) for all the prepared samples. The lyophilized samples were processed for coating with gold at 20 kV for SEM examination (Zeiss EVO 18 SEM Zeiss, Germany). In addition, Energy-Dispersive X-ray Spectroscopy (EDX) mapping was performed to investigate the elemental analysis of freeze-dried cow urine powder.

2.3 Thermogravimetric Analysis (TGA) Analysis of Freeze-Dried Cow Urine Powder

A thermogravimetric analytical instrument TGA-50 (Shimadzu (Asia Pacific) Pte Ltd.) was used to perform the Thermogravimetric Analysis (TGA). The variable range of temperature covering 20°C to 500°C with a heating rate of 10°C/min was optimized for experimenting. TGA helps in studying materials for their thermal stability by analyzing the weight change as a function of temperature, time, and atmosphere.

2.4 Differential Scanning Calorimetry (DSC) Analysis of Freeze-Dried Cow Urine Samples

The thermal analysis and behaviour of cow urine samples were assessed by carrying out DSC using an instrument DSC-60 Plus (Shimadzu [Asia Pacific] Pte.

Ltd). Measurements were taken under nitrogen gas at a flow and heating rate of 100 mL min^{-1} and 10°C min-1, respectively, beginning from room temperature to 300°C. The glass transition temperature and melting temperature was analyzed using the DSC thermograms obtained.

2.5 Nuclear Magnetic Resonance (NMR) Spectroscopy of Freeze-Dried Cow Urine Samples

The chemical structures of the major constituents of lyophilized cow urine powder were determined by ^{1}H and ^{13}C NMR spectroscopic measurements. ^{1}H and ^{13}C NMR spectra were monitored on a JEOL AL300 FTNMR (500 MHz) spectrometer at room temperature in either DMSO-d6 or CDCl3 solvent.

2.6 Fourier-Transform Infrared (FT-IR) Spectroscopic Analysis of Freeze-Dried Cow Urine Powder

FT-IR spectra were analyzed using a spectrometer (Thermo Scientific Nicolet iS5) with Potassium Bromide (KBr) pellets in the range of 400–4000 cm^{-1}. FTIR spectra were further analyzed to investigate the effect of the lyophilization process on the cow urine samples.

2.7 Cytocompatibility Study

In this study, we utilized mouse embryonic fibroblast NIH-3T3 cells to investigate the cytocompatibility of lyophilized cow urine powders. Dulbecco's Modified Eagle's Medium (DMEM) containing 10% Fetal Bovine Serum (FBS) and 1% Penicillin (10,000 U/ml)/ Streptomycin (10 mg/ml) (PS) was reconstituted as a complete culture medium to culture the cells in a CO2 incubator (Galaxy® 170 S, Eppendorf, Germany) maintained at a temperature of 37°C with the provision of 5% CO_2 and feeding of 95% relative humidity. Cells were cultured in a 96-well plate to study the effects of cow urine powders. Lyophilized cow urine powders were mixed at varying concentrations such as 0.5 mg/ml and 1.0 mg/mL in a complete culture medium to treat the cells. After 24 h treatment, cells were monitored through a bright-field microscope (Nikon Ti-U Eclipse, Japan) to observe the qualitative assessment of cellular morphology following the treatment of cow urine powders.

2.8 Assessment of Cellular Viability Using MTT Assay

MTT (3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide) reduction assay was performed to estimate the cellular viability. NIH-3T3 fibroblast cell was used for this purpose. The standard assay procedure was optimized to carry out the MTT assay using cells cultured in the 96-well plate.

A density of 10^4 cells per scaffold was used to culture them for 24 h in a CO_2 incubator. For all the experiments, a positive control was taken wherein cells cultured without any treatment were considered, while complete growth medium alone was treated as a negative control. Upon incubation of cells with freeze-dried cow urine powders, a solution containing MTT dye at the concentration of 5 mg/mL in PBS was prepared using 90 µL complete growth medium and 10 µL MTT solution and incubated with all the treated samples for 4 h. Formazan crystals formed by the cells were mixed in 100 µL volume of Dimethyl Sulfoxide (DMSO) at least for 15 min for its complete solubilization[18]. After mixing it gently, the mixed solution was further moved to a fresh multi-well plate after gentle mixing. A multimode reader was utilized to record the absorbance values set at a wavelength of 570 nm (Synergy H1 hybrid, Biotek, USA). MTT assays were performed in triplicates for all the cytocompatibility experiments.

2.9 Statistical Analysis

All the experimental analysis was carried out in triplicates and the outcomes were represented as mean ± Standard Deviation (SD). A one-way Analysis of Variance (ANOVA) method was used to examine the statistical significance of data along with Tukey's multiple comparison tests. All the data were compared and observed statistically different when $p<0.05$ was considered. OriginPro 2020 (OriginLab, Learning edition) was used further to plot all the graphs.

3. Results and Discussion

Cow urine is found effective in the treatment of colic, abdominal tumours, and enlargement of the abdomen (*Sushrut Samhita sutra sthan* 46/220-221). Cow urine

is also observed to be useful in the treatment of worms, leprosy, and itching (*Charak Samhita sutra sthan* 1/102). Cow urine has got a therapeutic value in the treatment of abdominal problems, flatulence, colic, anaemia, abdominal tumour, haemorrhoids, purgation, and sweating (*Astang Samgraha sutrasthan* 6/141-143). Owing to its high therapeutic value, cow urine has attracted much attention for further exploration and establishing its scientific merits. Although cow urine has traditionally been used especially in India in various forms such as distillate and lyophilized powder, its physicochemical properties and stability remain unexamined. In this study, we have attempted to establish the physicochemical characteristics of lyophilized powder of cow urine obtained from *Sahiwal* (Sample 1) and pure Indian breed (Sample 2).

3.1 FT-IR Spectroscopy

An infrared analysis was performed to understand the types of functional groups and their interactions at the molecule level within the lyophilized powder. FTIR spectra were analyzed in KBr pellets and shown in Figure 1. FTIR spectra of both samples showed a similar trend in band position and did not vary significantly. The FTIR spectra showed a broad peak at 3405 cm^{-1} in all the samples attributed to hydrogen-bonded O-H stretching frequency. The absorption peaks at 3065 cm^{-1}, 2940 cm^{-1} and 1735 cm^{-1} correspond to the aromatic ring C-H stretching, aliphatic chain C-H stretching and C=O stretching, respectively. The broad absorption peak at 1633 cm^{-1} wave number was observed in both the samples, which corresponds to amide I and II which attribute to the presence of urea. Absorption peaks at 1500 cm^{-1} and 1405 cm^{-1} correspond to aromatic C=C stretching.

3.2 NMR Spectroscopy

^{1}H NMR and ^{13}C spectra of both samples were analyzed in DMSO-*d6* and represented in Figure 2a-d. ^{1}H NMR spectra of all two samples did not differ significantly

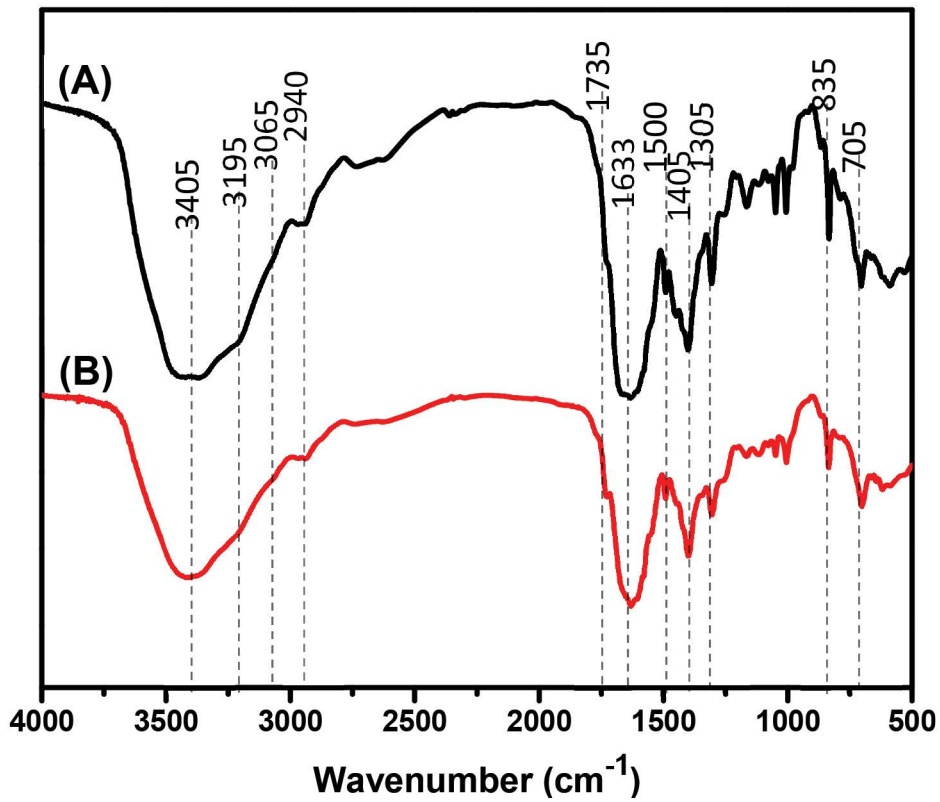
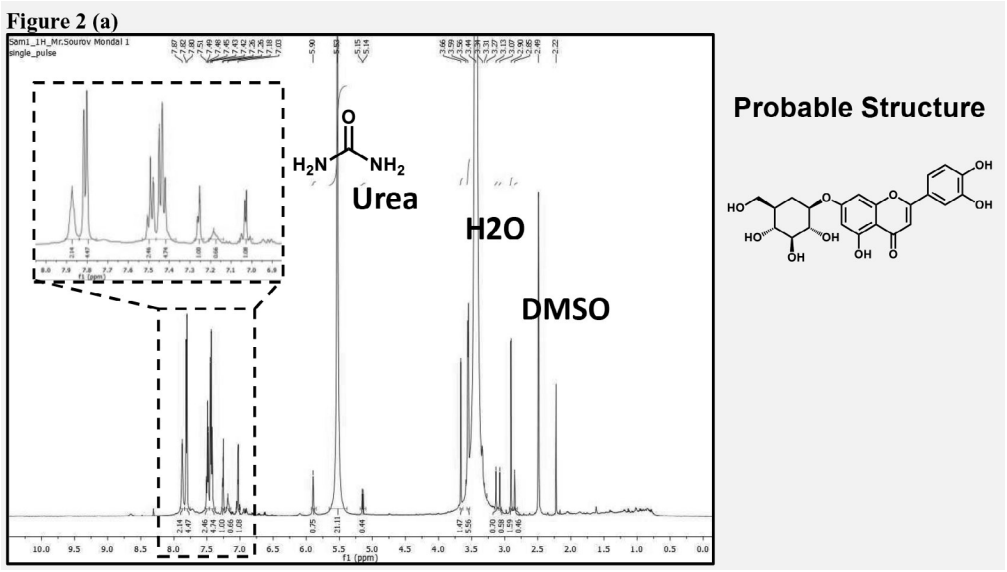


Figure 1. Fourier Transform Infrared (FT-IR) Spectroscopy of lyophilized cow urine. **(a)** Sample 1 (Cow urine obtained from Sahiwal breed). **(b)** Sample 2 (Cow urine obtained from pure Indian breed).

and showed a similar trend in peak position; confirming the presence of the same flavonoid/compounds in each sample. After analyzing peaks of NMR spectra, it can be confirmed that Luteolin-7-O-glycoside and urea are the major contents of lyophilized cow urine samples[19]. In addition, the integration of peaks at 5.53 ppm revealed that Sample 1 contained more amount of urea as compared to Sample 2.

3.3 SEM-EDX Analysis

Lyophilized powder of cow urine obtained from pure Indian breed (Sample 2) showed clusters-like morphology as can be seen in Figure 3(b). Whereas, lyophilized powder of cow urine obtained from *Sahiwal* (Sample 1) showed flex-like morphology containing clusters. The energy dispersive X-ray analysis



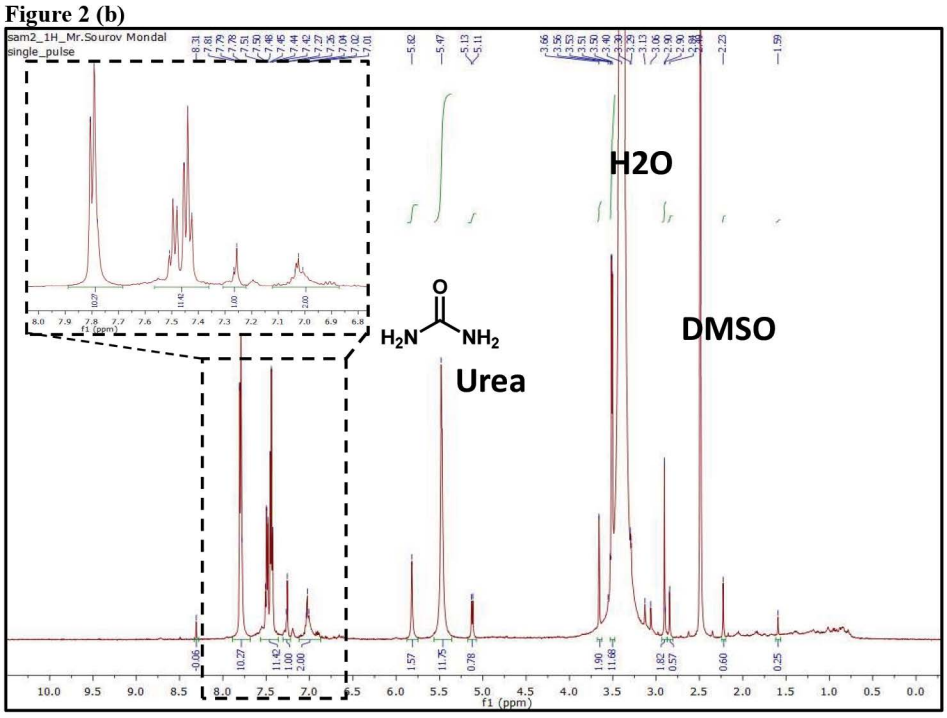


Figure 2. (Continued)

Figure 2 (c)

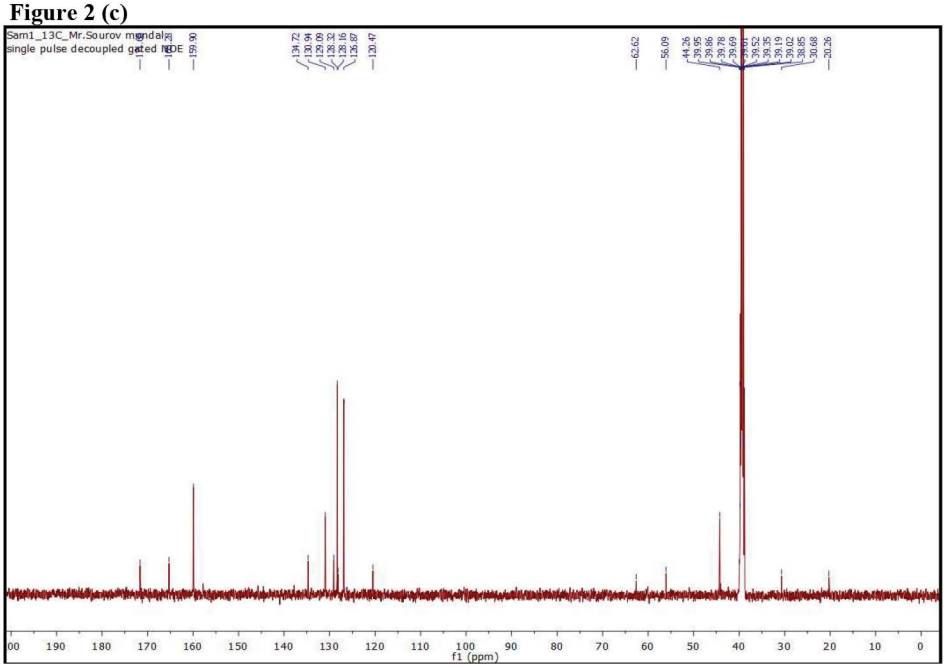


Figure 2 (d)

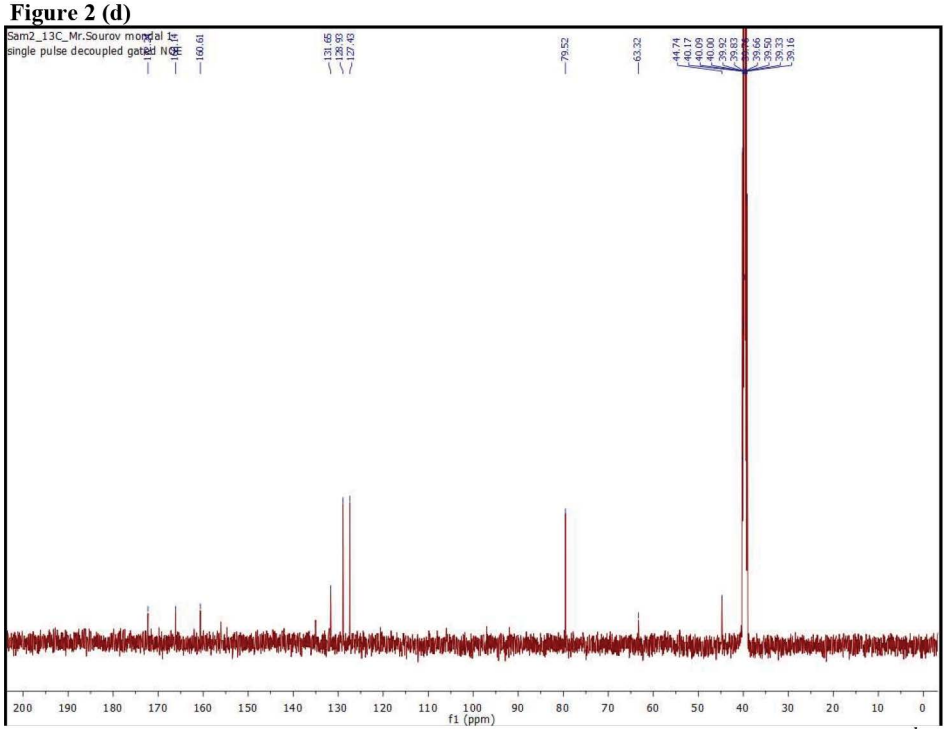


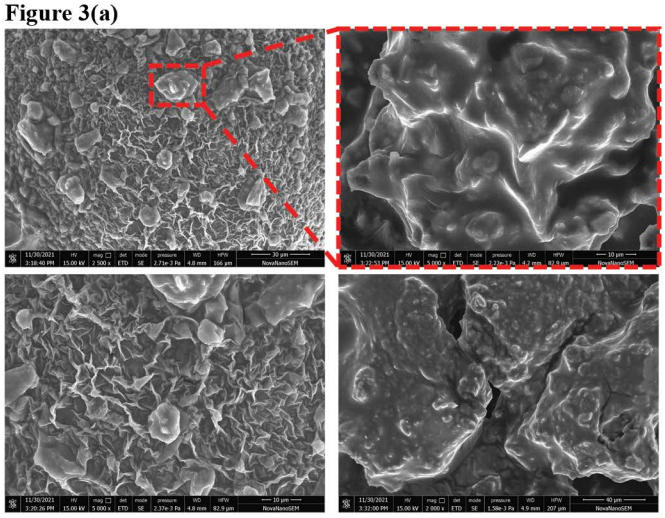
Figure 2. Nuclear Magnetic Resonance (NMR) spectroscopy of lyophilized cow urine. **(a)** ^{1}H NMR Spectra of Cow urine obtained from the Sahiwal breed. **(b)** ^{1}H NMR Spectra of Cow urine obtained from the pure Indian breed. **(c)** ^{13}C NMR Spectra of Cow urine obtained from the Sahiwal breed. **(d)** ^{13}C NMR Spectra of Cow urine obtained from pure Indian breed.

(EDX-mapping) revealed 25-30% of metals ion present in both samples. Sample 1 and Sample 2 contain 25% and 29% metal ions, respectively. The main elemental contents of the samples have been shown in Figures 3(a) and 3(b). The percentages of Gold (Au) and Silver (Ag) were found ranging 3.46-4.69% and 1.54-2.76%, respectively. The organic contents in samples 1 and 2 were found significantly varying approximately 24% and 71%, respectively.

3.4 TGA Analysis

Both samples showed three steps of degradation as shown in Figure 4. Initial weight loss from room temperature to 125°C attributed to desorption of adsorbed water. The higher weight loss percentage was found in the case of Sample 1 (7.6%) compared to Sample 2 (2.5%), which demonstrated more absorbed water on the surface of Sample 1. Thereafter, the next

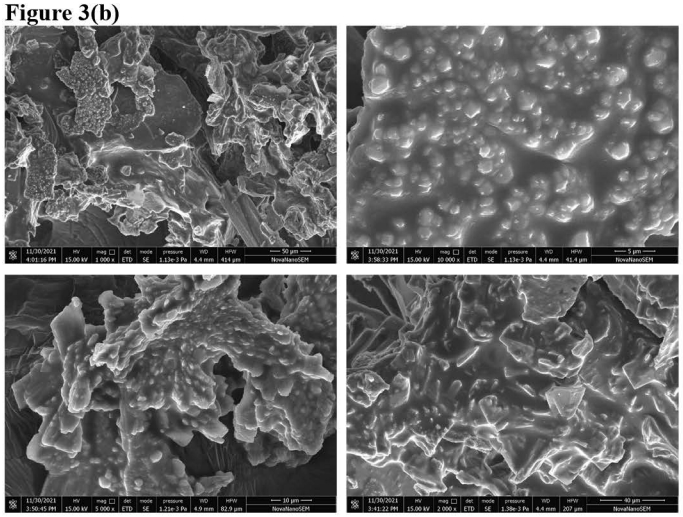
Figure 3(a)



Element	Weight %
C	19.72
N	3.38
O	10.23
Au	3.45
Mo	0.58
S	0.25
Pd	2.46
Ag	2.76
Ca	3.34
Mn	1.38
Fe	9.75

Approximate Organic 24%
Metals 25%

Figure 3(b)



Element	Weight %
C	29.57
N	13.59
O	28.58
Au	4.41
Cu	4.86
S	1.45
Pd	2.70
Ag	1.89
Ca	3.60
Mn	2.29
Fe	1.23

Approximate Organic 71%
Metals 29%

Figure 3. Scanning Electron Microscopy (SEM) with Energy-Dispersive X-ray spectroscopy **(**SEM-EDX) analysis of freeze-dried cow urine powder. **(a)** Sample 1 (Cow urine obtained from *Sahiwal* breed). **(b)** Sample 2 (Cow urine obtained from pure Indian breed).

degradation from 125-220°C in each sample was due to urea degradation present in cow urine. The present urea percentage was 19% and 15% in Sample 1 and Sample 2, respectively. Further, the next degradation of 230-340°C may be due to the loss of glycoside moiety present in flavonoid, i.e., Luteolin-7-O-glycoside. The glycoside loss percentage was 10.4% and 15% in Sample 1 and Sample 2, respectively. This represents that the resulting percentage of existing flavonoids in Sample 1 is higher than in Sample 2. After glycoside degradation, flavonoid ring degradation took place at the temperature range 370-800°C for Sample 1 and 340-800°C for Sample 2. The observed weight loss percentage was found to be 16% and 15% for Sample 1 and Sample 2, respectively. Overall, the remaining undegraded metal contents in Sample 2 (50%) is higher than in Sample 1 (42%).

3.5 DSC Analysis

From the data obtained and represented in Figure 5, we have found glass transition temperature (*T*g) lies nearly in the range of 110°C most likely due to the amorphous content of cow urine powder. Whereas, melting temperature (*T*m) lies in the range of 175°C possibly due to the crystalline content of cow urine powder. The results depict that the lyophilized powder of cow urine contains both irregular (amorphous) and ordered molecular structures (crystalline).

3.6 Cellular Compatibility of Lyophilized Cow Urine Powders

Cells treated with cow urine powders were found to have healthy morphology and were similar to those of controls. Post 24 h treatment, cells showed significant growth and no altered morphology over time (Figure 6). Both samples of cow urine exhibited similar cellular morphology following the treatment of the cells. Cells were more flattened and well adhered on the substrates. All the cow urine samples facilitated remarkable cell attachment and proliferation as observed from the captured images (Figure 6), while no noticeable difference in cellular morphology was found in different treatment groups. Thus, the outcomes suggest that the lyophilized cow urine powders are remarkably

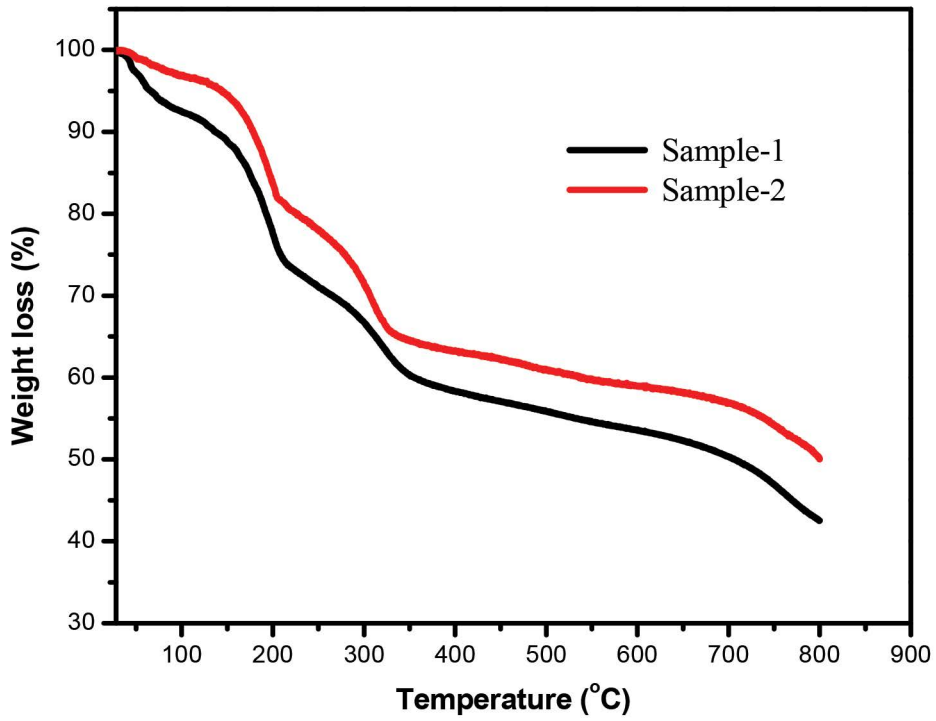


Figure 4. Thermogravimetric Analysis (TGA) of lyophilized cow urine. Sample 1 contains organic contents 58 % and metal contents 42 %. Sample 2 contains organic contents 50 % and metal contents 50 %. Sample 1: Cow urine obtained from the *Sahiwal* breed. Sample 2: Cow urine obtained from pure Indian breed

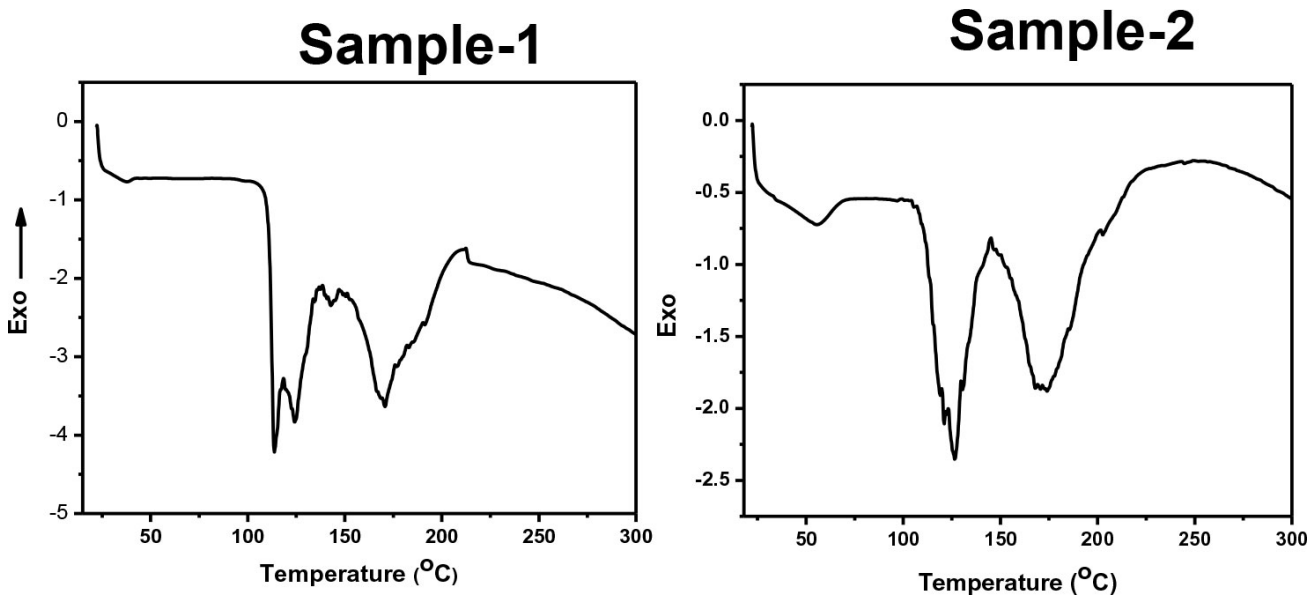


Figure 5. Differential Scanning Calorimetry Analysis (DSC) of lyophilized cow urine. **Sample 1:** Cow urine obtained from *Sahiwal* breed, **Sample 2:** Cow urine obtained from pure Indian breed

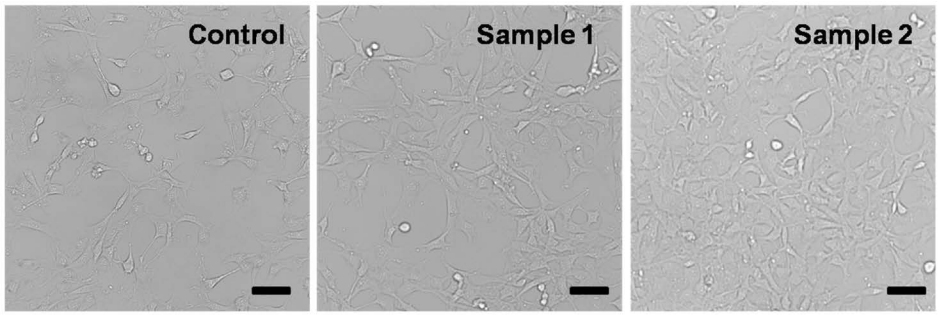


Figure 6. Cellular compatibility of lyophilized cow urine powders using NIH 3T3 fibroblast cells. **Sample 1:** Cow urine obtained from *Sahiwal* breed, **Sample 2:** Cow urine obtained from pure Indian breed

cytocompatible and significantly favour the process of cell adhesion and cell growth uniformly on the cultured plates. Overall, the outcomes reveal that lyophilized cow urine powders possess no deleterious effects on healthy fibroblast cells.

3.7 Quantitative Assessment of Cellular Viability Post-Treatment with Cow Urine Powders

Cells treated with cow urine powders were assessed using an MTT assay to determine the cellular proliferation of NIH-3T3 fibroblast cells following 24 h of treatment. No noticeable difference in the viability percentages of fibroblast cells was found following the treatment with lyophilized cow urine powders at both the concentrations of 0.5 mg/mL and 1.0 mg/mL (Figure 7). Similar observations were found in both samples of cow urine powders. Overall, the experimental observations indicate that the proliferation rate of fibroblast cells is significantly unaffected upon treatment with cow urine powders; inferring that cow urine powders possess no toxic effects on healthy fibroblast cells.

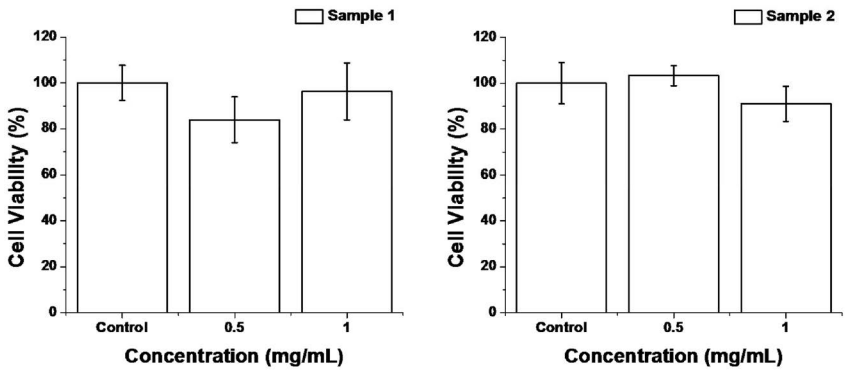


Figure 7. Effects of lyophilized cow urine powder on the viability of NIH 3T3 fibroblast cells estimated using an MTT assay. **Sample 1:** Cow urine obtained from *Sahiwal* breed and **Sample 2:** Cow urine obtained from pure Indian breed

4. Conclusions

Physicochemical properties of lyophilized powder of cow urine obtained from *Sahiwal* and pure Indian breed were examined through several analytical techniques, including Thermogravimetric Analysis (TGA), Fourier Transform Infrared (FTIR) Spectroscopy, Differential Scanning Calorimetry (DSC), Nuclear Magnetic Resonance (NMR) Spectroscopy and Scanning Electron Microscopy – Energy Dispersive X-ray Spectroscopy (SEM-EDX). Lyophilized powder of cow urine exhibited clusters-like morphology, the presence of 25-30% of metal ions, three steps degradation, and the presence of both irregular (amorphous) and ordered molecular structures (crystalline). ^{1}H NMR spectra and FTIR spectra showed similar trends and did not vary significantly; confirming the presence of the same flavonoid/compounds in each sample. In addition, cytocompatibility study and MTT assay revealed that lyophilized cow urine powders possess no toxic effects on healthy fibroblast cells. We anticipate that the outcomes could be potentially useful for analyzing the physicochemical stability, cytocompatibility, and therapeutic value of the lyophilized form of cow urine.

5. Acknowledgements

Pallawi would like to thank Banaras Hindu University (BHU) for providing her fellowship and contingency support. The authors gratefully acknowledge the Indian Institute of Technology (Banaras Hindu University) for providing access to the Central Instrumentation Facility for carrying out the analytical characterization.

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