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FT-IR and GC-MS analyses of potential bioactive compounds of cow urine and its antibacterial activity

Vipin Nautiyal *, R.C. Dubey

Department of Botany and Microbiology, Gurukula Kangri (Deemed to be University), Haridwar – 249404, Uttarakhand, India

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ABSTRACT

The main emphasis of this study was to identify the bioactive compounds responsible for antibacterial activity of *Badri* cow urine isolated by thin layer chromatography. The most effective bioactive fraction was analysed by FT-IR and GC-MS analyses. Among the four major fractions (EW1, EW2, CA1 and CA2) obtained by TLC profiling, EW1 was found most active against bacterial strains viz., *Listeria monocytogenes* (MTCC657), *Staphylococcus aureus* (MTCC7443), *Pseudomonas aeruginosa* (MTCC424), *Klebsiella pneumoniae* (MTCC432) and *Salmonella typhi* (MTCC733). However, *Escherichia coli* (MTCC118), was found resistant to all the fractions. In FT-IR spectroscopy, functional groups like alcohol, amide, alkene, alkyl halide, polysulfide and phosphate ions were identified. The GC-MS analysis of EW1 fraction exhibited the presence of 12 compounds, of which 1-heneicosanol was found as the major compound. These compounds might be responsible synergistically or individually for antibacterial activity of cow urine. Nine elements namely sodium (Na), calcium (Ca), chromium (Cr), iron (Fe), magnesium (Mg), aluminium (Al), potassium (K) and zinc (Zn), Gold (Au) were measured by ICP-MS analysis.

1. Introduction

In the developing countries like India, the indigenous sources of drugs have been explored to get new and more therapeutically active compounds. These indigenous substances have numerous advantages over the conventional drugs and therapies. The emergence of bacterial resistance to antibiotics is one of the serious health concerns across the world. The new emerging multi-drug resistant (MDR) strains cause mortality and morbidity in the immunocompromised patients (Nascimento et al., 2000). The futility of antibiotics is mainly due to its irrational use for treatment of various ailments as well as in livestock sector and lack of awareness among the medical practitioners (Davies and Davies, 2010). Due to global recognition of natural resources in medicine, it is very important to expand the knowledge and research towards unique traditional system of medicine.

The cow urine is widely known to possess various pharmacological properties as described in Ayurvedic texts like *Sushruta Samhita* and *Ashtanga Sangraha*. In Indian scriptures, cow is considered as 'Kamdhenu', means the "mother of all entities". *Panchgavya* (mixture of cow urine, dung, milk, curd and ghee) is very popular among all the major ayurvedic formulations and used predominantly for religious as well as therapeutic purposes. It showed antibacterial activity against human pathogenic bacterial strains namely *Staphylococcus aureus*, *Bacillus cereus*, *Salmonella typhimurium* and *Escherichia coli* (Kishore et al., 2019). The major population of hilly states of India, like Uttarakhand and Himanchal Pradesh, still rely on cows for its economy, survival and traditional remedy. For example, the combination of lichen (*Peltigra polydactyla* Hoffm.) and cow urine is used in Himanchal Pradesh in the form of antiseptic over ruptured skin as traditional remedy (Sharma, 1997). Furthermore, the combination of cow urine and medicinal plants is also used to prepare biodegradable, cost-effective and eco-friendly disinfectants.

Cow urine consists of mainly iron, copper, nitrogen, sulphur, manganese, carbolic acid, chlorine, magnesium, citric, calcium salts, enzymes, mineral salts, creatinine, uric acid, hormones and vitamins (A, B, C, D and E) (Vinay et al., 2019). It also enhances the bioavailability of certain drugs (Nishanth et al., 2010). The antibacterial activity of cow urine against many pathogenic bacteria was reported by several researchers (Singh et al., 2012; Rakesh

et al., 2013; Nautiyal and Dubey, 2020). However, literatures available on the identification of bioactive compounds are scanty and meagre. Therefore, the present study was aimed to identify the active components of *Badri* cow urine using FT-IR and GC–MS profiling and assess its antibacterial properties.

2. Material and methods

2.1. Collection of Badri cow urine

Badri cows are housed in Cattle Breed Centre, Nariyalgaon, Champawat district (29°20′N latitude and 80°5′E longitude) of Uttarakhand state. Early morning mid-stream urine sample was collected using a sterile airtight container and brought to the laboratory. It was filtered by filter paper (Whatman No.1) to get rid of precipitates.

2.2. Chromatography analysis

Thin layer chromatography (TLC) system was used to separate different bioactive compounds present in cow urine. 10 μL of urine was spotted by a capillary tube on precoated silica gel chromatography plates (Merck, TLC grade) as stationary phase, whereas the different solvent systems were used as the mobile phase. The best separation was achieved by using solvents ethanol/water (80:20 v/v) and chloroform/acetic acid (80:20 v/v). After the development of chromatograms, the plates were removed and allowed to dry and remove the mobile phase. The position of different compounds on plate was visualized under UV light (Vilber Lourmat). The retention factors (Rf) were calculated for each spot using the following formula:

$$Rf = \frac{\text{Distance travelled by solute}}{\text{Distance travelled by solvent}}$$

2.3. Evaluation of antibacterial activity

2.3.1. Bacterial strains
The bacterial strains namely, *Listeria monocytogenes* (MTCC657), *Staphylococcus aureus* (MTCC7443), *Pseudomonas aeruginosa* (MTCC 424), *Escherichia coli* (MTCC 118), *Klebsiella pneumoniae* (MTCC 432) and *Salmonella typhi* (MTCC 733) were procured from the Microbial Type Culture Collection (MTCC), Chandigarh (India). All the bacterial strains were sub-cultured at regular intervals on Luria Bertani (LB) slants. The slants were stored at 4 °C for further use.

2.3.2. Inoculum preparation
The bacterial strains were inoculated in freshly prepared LB broth and incubated at 37 °C for 24 h. The turbidity was then adjusted to 0.5 McFarland standard (1 x10⁸ CFU).

2.3.3. Agar well diffusion assay
Agar well diffusion assay was carried out as per the method of Holder and Boyce (1994). All the bacterial strains were spread on the surface of respective plates containing freshly prepared nutrient agar medium (NAM) by sterile cotton swab and allowed to dry. Wells (6 mm diameter) were punched by a sterile cork borer and 50 μL of suspension of different fractions was dispensed into the wells. Thereafter, all the plates were incubated at 37° C for 24 h and zone of growth inhibition was measured. On the basis of antibacterial activity, the potential fraction was used for further study.

2.4. Fourier transform infrared spectroscopy (FTIR) analysis

The FTIR spectra of bioactive fraction of cow urine was obtained in FTIR instrument (Perkin Elmer, spectrum ™ 400) with data processing unit. Small amount of sample to be analysed was made into pellets by using potassium bromide (KBr IR grade) and a thin film was prepared by applying pressure. The infrared transmittance spectrum was recorded over a wave number ranging from 4000 cm^{-1} to 400 cm^{-1}. The spectral data were compared to reference in order to identify the chemical bond and functional groups existing in the sample (El-Naggar et al., 2017).

2.5. Gas chromatography – Mass spectrometry (GC–MS) analysis

The fraction (EW1) was subjected to GC–MS analysis using Thermo Trace 1300GC coupled with Thermo TSQ 800 Triple Quadrupole MS equipped with Column (BP 5MS 30 m X 0.25 mm, 0.25 μm). The initial temperature of oven was 60℃ and increased finally to 230℃ by 5℃/min rate. Helium at flow rate of 1 mL/min was used as carrier gas. The injection volume was 2.0 μL (split ratio of 5:1) while the injector temperature was maintained at 250℃ and the ion source temperature was 230℃. The scan mass range of m/z 40–700 and the identification of existing components was done by comparing their mass spectra with standard data of NIST (National Institute of Standards and Technology) library (Ashraf et al., 2017).

2.6. Inductively coupled Plasma-Mass spectrometry (ICP-MS) analysis of Badri cow urine

2.6.1. Instrumentation
The elemental analysis was done with an ICP-MS/MS instrument (8900 ICP-MS/MS, Agilent) equipped with quadrupoles. Helium was used as the reaction gas in order to minimize spectral interference. The instrument composed of an automatic sampler, a double pass spray chamber and a concentric nebulizer. The ICP-MS instrumental operation setting used in this study are listed in Table 3.

2.6.2. Sample preparation
The elemental analysis of cow urine was done by ICP-MS. Cow urine was centrifuge at 3500 rpm for 15 min and the supernatant was filtered thrice by using Whatman No.1 filter paper. Then, 2 mL of cow urine was mixed with 8 mL of 2% nitric acid (Trace Metal Ultrapure Grade from Fisher Scientific) and heated gently to digest the elements. The volume of solution was maintained by addition of 2% nitric acid. The solution was again filtered and the filtrate volume was made up by addition of deionized water.

2.6.3. Standard solution for ICP-MS
The multi-element determinations were used for standard. Ge, Rh and Pt (1000 mg L^{-1}) was used as internal standard. The solutions were prepared by using nitric acid (ultrapure traceable grade, Fisher scientific) and deionized water. The external standard calibration method was adopted for each analyte determination. All the solutions were prepared in 2% HNO₃ (v/v).

3. Results

The TLC of cow urine was performed for the separation and purification of compounds present in urine. The fractions were abbreviated as EW1, EW2, CA1 and CA2, and the Rf values of these fractions were 0.59, 0.53, 0.83, 0.69, respectively. The plates were observed under UV light. The developed fractions were scrapped off and dissolved in methanol for the determination of antibacterial activity.

The EW1 posed the maximum inhibitory activity against *S. aureus* (15 mm ZOI) followed by *K. pneumoniae* (7 mm ZOI) among the all fractions, while the other fractions were less effective to bacterial strains (Figs. 1 and 2). The effect of all the four fractions was more or less similar against *L. monocytogenes*, *P. aeruginosa*, *S. typhi* and *E. coli*. But *E. coli* was not affected by the biochemicals present in any of the fractions. The fractions posed the maximum inhibitory activity against Gram-positive bacteria as compared to Gram-negative bacterial strains. Because of having the maximum antibacterial activity, the EW1 fraction was potent fraction, hence used for further analysis.

FTIR spectroscopy of bioactive fraction of EW1 fraction of cow urine was done to determine the infrared spectrum of the absorption of the compound as well as to identify the functional groups of compounds. The functional groups were separated on the basis of their peaks. The FTIR spectrum was recorded between 4000 and 400 cm^{-1} (Fig. 3). The FTIR analysis showed the presence of intramolecular bonded alcohol, amide, phosphate ion, strong and medium alkene bending, alkyl halide and polysulfide stretch (Table 1).

The identification of various chemical components present in EW1 fraction of cow urine was determined by GC–MS analysis (Fig. 4). The major compounds with their respective RT, peak area, molecular formula, and molecular weight are shown in Table 2. Identification of bioactive compounds in EW1 was done by comparing the obtained spectral peak with their respective reference compounds in NIST (National Institute of Standards and Technology) Library. The major compounds identified by GC–MS were fatty alcohols; 1-heneicosanol (37.91%), n-heptadecanol-1 (19.05%), n-nonadecanol-1 (17.0%) and 1-hexadecanol (0.752%). Other components included pentadecanal (2.80%), 1,1,1,3,5,7,7,7, octamethyl-3–5 bis (trimethyl siyloxy) tetra siloxane (2.35%), cyclooctasiloxane, hexadecamethyl (1.70%), 6H-Pyrazolo[1,2-a][1,2,4,5]tetrazine, hexahydro-2,3-dimethyl- (0.47%), 2(1H)-Pyrimidinone, 5-chloro-4,6-diphenyl (1.60), 1-triethylsilyloxyheptadecane (1.10%), 1,4-dioxane-2,6-dione (0.51), 2-pentanone, 4-hydroxy-4-methyl- (0.87) were also identified.

Nine biologically important inorganic elements such as Na, Ca, Cr, Fe, Mg, Al, K and Zn, Au were measured by ICP-MS analysis that were present at varying concentrations (Table 4). Potassium was present at higher amount followed by Mg, Na, Ca, Zn, Fe, Al, Cr and Au.

4. Discussion

All the four fractions of *Badri* cow urine separated on TLC were isolated and its antibacterial activity was measured. EW1 was most potential among all the four fractions because of the presence of certain inhibitory compounds in EW1 fraction that might be responsible for their antibacterial activity. The inhibitory effect of cow urine may be due to the presence of some volatile and non-volatile compounds (Shaw et al., 2007). Further, FT-IR analysis of EW1 confirmed the presence of alcohol, amide, phosphate ion, alkene, alkyl halide and polysulfide groups. Widiyanto et al. (2014) have also found the presence of amide, amine and CH$_3$ bending in cow urine.

GC–MS analysis of bioactive fraction of cow urine identified the presence of 12 components at varying concentrations at specific retention time. The components were separated on the basis their mass/charge ratio at a particular RT. The presence of long chain fatty alcohol; 1-Heneicosanol at RT 36.37 with maximum peak area 37.91% followed by n-Heptadecanol-1 and n-Nonadecenol-1 at RT 28.05 and 33.80 with peak area 19.05% and 17.0%, respectively were noticed in this study. The fatty alcohols are known to exhibit significant role in biological activities. Fatty alcohols might be responsible for the antibacterial activity of bioactive fraction of cow urine. Togashi et al. (2007) evaluated the antibacterial activity of long chain fatty alcohol against *S. aureus* and reported the inhibitory effect by induction of K$^+$ ion leakage from bacterial cell membrane. Chatterjee et al. (2018) proved the antibacterial activity of long chain alcohols against *Salmonella gallinarum*. Moreover, the presence of fatty acid like hexadecanoic acid or palmitic acid and *trans*-9-octadecanoic acid in camel urine was confirmed by Ahamad et al. (2017). Begum et al. (2016) isolated ruminant microflora, *Paracoccus pantotrophus* from ruminal fluid and identified the fatty alcohols and other active compounds as bacteria-derived metabolites by GC–MS analysis. The fatty acids are considered as a chief source of energy for ruminants which are produced during the fermentation process in rumen by rumen microflora
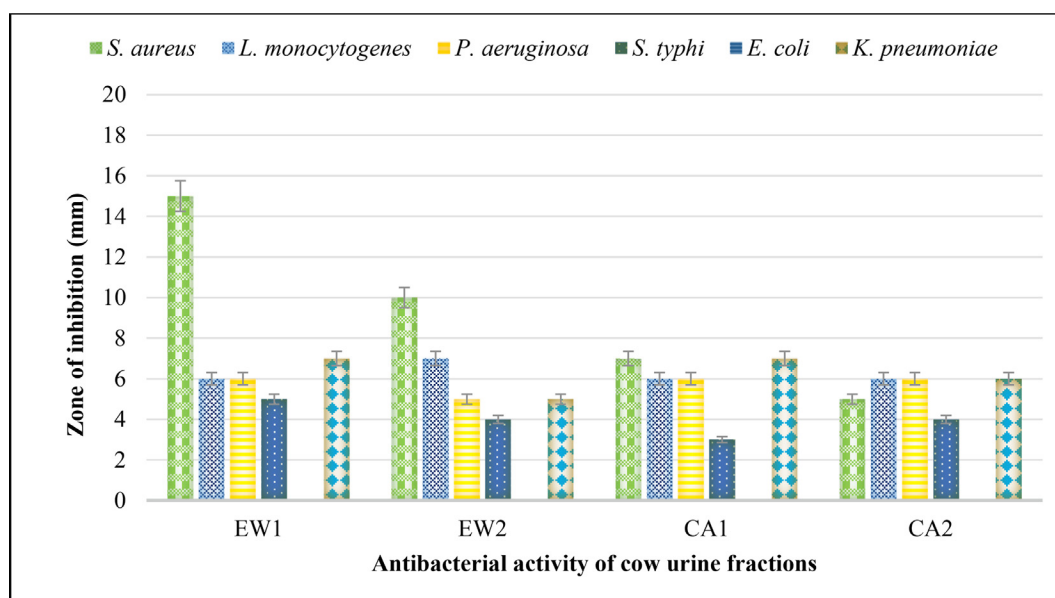

Fig. 1. Antibacterial activity of cow urine fractions.

Fig. 2. Antibacterial activity of different fractions against various bacterial strains.
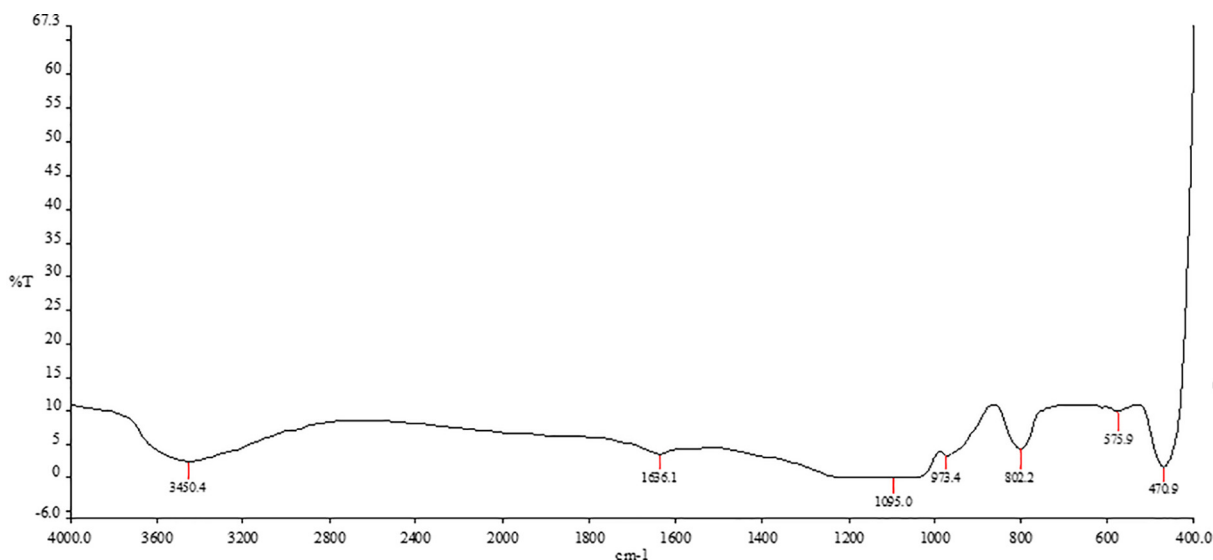

Fig. 3. FT-IR spectrum of EW1 fraction.

(Erwin et al., 1961). In our study, the presence of long chain fatty alcohols was found in higher concentration as compared to other compounds, which might be responsible for antibacterial activity. Pentadecanal (fatty aldehyde) was also present in EW1 fraction which was found active against biofilm producing *S. epidermidis* (Ricciardelli et al., 2018).

Among siloxanes 1,1,1,3,5,7,7,7, octa methyl-3–5 bis (trimethyl siyloxy) tetra siloxane, cyclooctasiloxane, hexadecamethyl and 1-triethylsilyloxyheptadecane having peak area 2.35%, 1.70% and

1.10%, respectively were identified as major contributors of bioactive fractions. Moreover, several other compounds like 6H-pyrazolo[1,2-a] [1,2,4,5] tetrazine, hexahydro-2,3-dimethyl-, 2 (1H)-pyrimidinone, 5-chloro-4,6-diphenyl, 1,4-dioxane-2,6-dione and 2-pentanone, 4-hydroxy-4-methyl were also detected.

The inorganic elements are essentially needed for normal biological function of human body (Ahamad et al., 2017). Potassium is an essential mineral found most abundantly in human body and mainly located in intracellular fluid where it maintains vital

Table 1
FTIR peak assignment of analysed fraction compared with standard chart.

Peaks	Functional groups
3450.4	Intramolecular bonded O-H stretching
1636.1	C = N stretching amide
1095.0	PO_2^- stretching phosphate ion
973.4	Strong C = C bending alkene
802.2	Medium C = C bending alkene
575.9	Strong C-Br stretching alkyl halide
470.9	Polysulfides S-S stretching

cellular functions (Ekmekciogl et al., 2016). In the present study, potassium was also present in highest amount as compared to other analysed elements. It involves in many important biological functions like carbohydrate metabolism, insulin secretion and protein synthesis (Ringer and Bartlett, 2007). In addition, Na is another crucial extracellular cation which regulates the distribution of water and osmotic pressure in body fluids (Naik and Maben, 2018). Low Na intake or its deficiency leads to many health-related concerns. The relationship between Na-K ratio with cardiovascular disease has been established (Cook et al., 2009). Moreover, Cr is also an important mineral that is involved in human metabolic pathways. Chromium supplement is beneficial in diabetes and lipid disorders (Nussbaumerova et al., 2018).

Iron plays a significant role in carbohydrate, protein, lipid and nucleic acid metabolism. More importantly, one of the forms of iron is called heme iron attached with porphyrin and present in the prosthetic group of haemoglobin as well as myoglobin. On the other hand, non-heme iron binds with low molecular weight chelating agents (Milto et al., 2016). Furthermore, Zn and Ca are also equally important for normal biological function. The considerable amount of Mg was measured in *Badri* cow urine. Magnesium (Mg^{2+}) is known as one of the most abundant divalent cations found in prokaryotes as well as in eukaryotes and perform many physiological functions, such as growth and development (Dudev and Lim, 2013). It acts as cofactor for many enzymatic reactions in different cellular functions. It is also involved in carbohydrate metabolism as rate-limiting enzymes (Yang et al., 2014) and in lymphocyte synthesis and its proliferation (Forte et al., 2005). The presence of such elements at different levels in cow urine has also been confirmed by Ahamad et al. (2017). Ours is the first report on the presence of such compounds and elements in *Badri* cow urine.

5. Conclusion

The present study highlights the presence of different bioactive compounds and micro- and macro-elements in cow urine having antibacterial activity. The FT-IR and GC–MS analyses of EW1 fraction clearly showed the presence of long chain fatty alcohols as
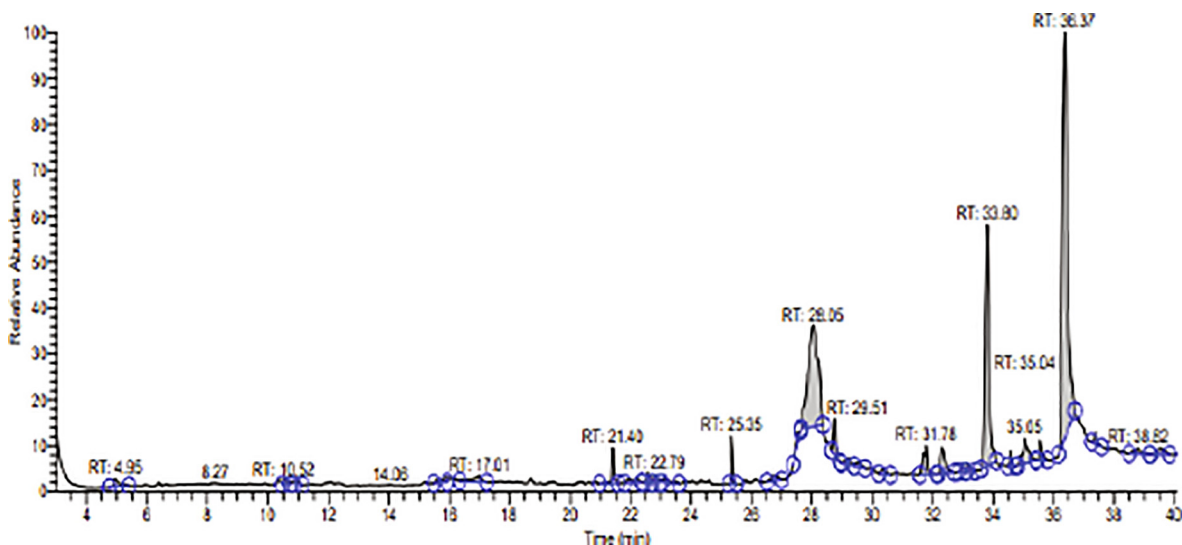
Fig. 4. GC–MS chromatogram of EW1 fraction.

Table 2
GC–MS analysis of EW1 fraction of cow urine.

Compound name	Retention time	Molecular formula	Molecular weight	Area (%)
1-Heneicosanol	36.37	$C_{21}H_{44}O$	312.6	37.91
Pentadecanal	35.04	$C_{15}H_{30}O$	226.4	2.80
n-nonadecanol-1	33.80	$C_{19}H_{40}O$	284.5	17.0
1,1,1,3,5,7,7,7, octa methyl-3–5 bis (trimethyl siyloxy) tetra siloxane	31.78	$C_{14}H_{42}O_5Si_6$	458.99	2.35
1-Hexadecanol	29.51	$C_{16}H_{34}O$	242.44	0.752
n-Heptadecanol-1	28.05	$C_{17}H_{36}O$	256.5	19.05
Cyclooctasiloxane, Hexadecamethyl	25.35	$C_{16}H_{48}O_8Si_8$	593.2	1.70
6H-Pyrazolo[1,2-a][1,2,4,5]tetrazine, hexahydro-2,3-dimethyl-	22.79	C7H16N4	156.23	0.47
2(1H)-Pyrimidinone, 5-chloro-4,6-diphenyl	21.40	$C_{16}H_{11}ClN_2O$	282.72	1.60
1-Triethylsilyloxyheptadecane	17.01	$C_{23}H_{50}OSi$	370.7	1.10
1,4-Dioxane-2,6-dione	10.52	$C_4H_4O_4$	116.07	0.51
2-Pentanone, 4-hydroxy-4-methyl-	4.95	$C_6H_{12}O_2$	116.15	0.87

Table 3
Instrumental setting for ICP-MS analysis.

Instrument	Agilent ICP-MS 8900
RF Power	1550 Watts
RF matching	1.40 V
Sample depth	8.0 mm
Plasma gas flow	15.0 L/min.
Auxiliary gas flow	0.90 L/min.
Plasma mode	Low matrix
Nebulizer gas flow	1.03 L/min
Nebulizer pump	0.10 rps
S/C Temperature	2 °C
Number of replicates	3

Table 4
Inorganic constituents of cow urine detected by ICP-MS.

Name of element	Element level (ppb)
Sodium (Na)	7605.901
Calcium (Ca)	1311.903
Chromium (Cr)	0.187
Iron (Fe)	19.555
Magnesium (Mg)	24836.975
Aluminium (Al)	7.555
Potassium (K)	3230193.196
Zinc (Zn)	81.308
Gold (Au)	85.34

principal component. This study suggests the possible use of these bioactive compounds against various ailments and supports the application of *Badri* cow urine mentioned in holy texts '*Vedas*'.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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