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Research Article

EVALUATION OF ANTIMICROBIAL ACTIVITY, GC-MS AND FT-IR PROFILE OF COW URINE DISTILLATES PREPARED FROM FRESH URINE OF INDIGENOUS COW BREEDS

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ABSTRACT: The present study was undertaken to explore the antimicrobial potential of cow urine distillate (gomutra ark) against pathogenic microorganisms of medical and veterinary significance. Cow urine distillates (CUDs) were prepared from fresh cow urine of three Indian breeds *viz.* Sahiwal, Tharparkar, and Vrindavani. CUDs obtained from two goshalas and one commercially available CUD were also tested for antimicrobial activity for comparison. Minimal inhibitory concentration (MIC) of CUD, antimicrobial activity of concentrated CUD, bacteriostatic and bactericidal potential as well as vapor phase activity were also determined. FT-IR and GC-MS analysis of the selected CUDs was performed to detect the surface functional groups and chemical composition. No antimicrobial activity was detected in the CUDs by disc diffusion and agar well diffusion methods. However, antimicrobial activity was observed in the microtitre Plate (MTP) assay as evidenced by inhibition of visible growth (turbidity) or measuring optical density (OD595). CUDs prepared from indigenous cows (Sahiwal and Tharparkar) exhibited superior antimicrobial activity compared to CUDs from crossbred cows (Vrindavani) or commercial CUDs. In general, CUDs were bactericidal for Gram-negative bacteria but bacteriostatic for Gram-positive bacteria. The MIC of CUDs was found to be in the range of 1:4 to 1:8 dilution. The concentrated CUDs exhibited a relatively lesser antimicrobial effect. CUDs exhibited antifungal activity against *Candida albicans* and *Malassezia furfur*. CUDs also exhibited antimicrobial activity in the vapor phase. FT-IR spectra of selected CUDs exhibited bending vibrations at 1633.5 cm-1 due to N-H groups resembling N-H bonding in the structure of urea and stretching vibrations at 3330. 10 cm-1 due to C-H group. GC-MS analysis of selected CUDs exhibited many compounds that may be responsible for the antimicrobial activity. The results of the study confirm the antimicrobial potential of CUD as reported in ancient literature.

Keywords: Antimicrobial activity, Cow urine, Cow urine distillate, Antibacterial, Antifungal, GC-MS.

INTRODUCTION

The cow has been regarded as a sacred and most valuable animal in the Vedas [1, 2]. Cow urine has been used as a medicine since ancient times and is recommended to improve health in Ayurveda. In Ayurvedic scriptures *viz.* Ashtanga Sangrah, Bhav Prakash Nighantu, and Sushruta Samhita, the cow urine (gomutra) has been defined as a therapeutic secretion of animal origin effective against various diseases such as AIDS, cancer, diabetes, diarrhea, edema, jaundice, and tuberculosis [3, 4, 5]. Cow urine has also been reported to cure hemorrhoids, anemia, and vitiligo and carry an antioxidant activity [6, 7, 8]. Cow urine comprises 95% water, 2.5% enzymes, 2.5%

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urea, hormones, salts, and minerals like iron, phosphorus, nitrogen ammonia, manganese, sulfur, potassium, and amino acids [9]. Studies reveal that cow urine contains lactose, peptides, and vitamins A, B, C, and D. Cow urine also contains volatile and non-volatile components which are responsible for antimicrobial properties [10, 11, 12]. Additionally, creatinine, phenols, certain urinary peptides, and urea may be responsible for the antimicrobial properties of cow urine [13, 14].

Cow urine distillate (CUD) is the purified form of cow urine which is prepared by heating the urine and collecting the condensed vapor. CUD has been reported to exhibit antimicrobial, antioxidant, and anti-cancerous properties [15, 16, 17]. Studies reported that CUD inhibited the growth of both Gram-negative and Gram-positive bacteria including *Bacillus subtilis*, *Staphylococcus aureus*, *Streptococcus* spp., *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus vulgaris*, *Pseudomonas* spp., and *Salmonella* Typhi [5, 17]. CUD has been reported to retard the growth of fungal pathogens including *Aspergillus niger, A. flavus, A. fumigatus, Rhizopus* spp*., Candida ablicans, C. tropicalis,* and *C. glabrata* [18, 19, 20]. In addition, CUD has also been reported to act as a bio-enhancer for antifungal, antibacterial, and anticancer components [18] and consequently granted a US patent for bio-enhancing properties for antibiotics and anticancer agents [21]. CUD from indigenous cows has been shown to induce a higher level of bio-enhancement and body immunity because of its '*Rasayanatatva*' [22].

Regardless of the tremendous growth of human medicine, infectious diseases still are a major threat to public health. The development of antimicrobial resistance is a part of the struggle for existence of the microorganisms and it reached a dangerous stage presently [23, 24, 25]. Due to the irrational use of antimicrobials, the problem is strengthened. Even various animal products are found with resistant microorganisms [26, 27]. Currently, the fifth generation of antibiotics is in use but the microbes have acquired resistance to every generation of available antibiotics, even before the introduction of them to the market [24]. The death that occurred due to antimicrobial-resistant (AMR) bacteria has been estimated to be around 0.7 million per year which is predicted to rise to 10 million people per year by 2050 and is estimated to cost about 100 trillion USD to the global economy [23]. This situation is alarming and further intensified due to a substantial drop in the rate of antibiotic discovery. The current antibiotic discovery pipeline is dry as no new class of antibiotics has been discovered to combat the antibiotic resistance bacteria which has led to fears of entering into to 'post-antibiotic era' [28, 29]. Therefore, there is a need to explore various alternatives to control drug-resistant infections. Since AMR has become a serious global concern, the need for new treatment modalities is urgent. Moreover, the resistant nature of bacteria against the available regime of antibiotics has increased the demand for new and natural antimicrobial agents [5].

Although several reports of the medicinal activity of CUDs on different pathogenic microorganisms have been published by various researchers [30], still there is a need to scientifically evaluate the claimed antimicrobial properties of CUD. Therefore, the present study was carried out to evaluate the antimicrobial properties of CUDs produced in-house from freshly collected urine of indigenous cow breeds.

MATERIALS AND METHODS

Collection of fresh urine and production of cow urine distillate (CUD)

Fresh urine was collected from three indigenous breeds of cows; Sahiwal (S), Tharparkar (T), and Virandavani (V) from the Livestock Farm of ICAR-Indian Veterinary Research Institute (ICAR-IVRI), Izatnagar, Bareilly, (UP). Urine was collected during morning time in a sterile container and brought to the laboratory. Urine was filtered through Whatman filter paper No. 1 to remove debris (if any). Cow urine distillate (CUD) was prepared using a cow urine distillation unit (Standard Scientific Glass Industries, Mumbai, India) available at Food Microbiology Laboratory, Division of Livestock Products Technology, ICAR-Indian Veterinary Research Institute, Izatnagar 243122 Bareilly (UP), India. Urine was filled in the glass reservoir and allowed to heat for distillation at a constant temperature (60ºC) and the vapors formed were condensed, collected in a sterile glass bottle, and stored in the refrigerator at 4±1ºC for further study. A total of 14 CUDs were produced (06 Sahiwal; 06 Tharparkar and 02 Vrindavani). Two prepared CUDs were procured from Goshalas (Kanerimath and Shahjanpur) and one commercially available CUD from the local market was also included in the study for comparison.

pH and mineral composition of CUD

The pH of CUDs was measured using a digital pH meter (Labman, India). The presence of zinc, copper,

iron, and cobalt was determined by atomic absorption spectroscopy (Shimadzu, Japan). Calcium was measured as per the methodology of Talapatra *et al.* (1940) while phosphorus was measured as per the methodology of AOAC (2012) [41, 42].

Antimicrobial activity of CUD

Microorganisms

Three Gram-positive, *viz. Bacillus cereus* (ATCC10876), *Listeria monocytogenes* (ATCC656), *Staphylococcus aureus* (ATCC29213) and five Gram negative *viz. Escherichia coli* (C-338), *Escherichia coli* (C-173), *Salmonella* Typhimurium (MTCC-3231), *Citrobacter freundii* (MTCC2956), and *Yersinia enterocolitica* (ATCC23715) bacterial strains were used for antibacterial study. A total of five fungal strains *viz. Candida albicans* (ATCC 10231), *Malassezia furfur* (12345), *Aspergillus fumigatus* (ATCC 204305), *Trichophyton mentagrophytes* (ATCC 9533), and *Trichophyton rubrum* (ATCC 28188) were used to evaluate the antifungal activity. Microbial cultures were selected to represent Gram-positive and Gram-negative branches in the case of bacteria and both yeast and mold forms in the case of fungus. Microbial strains selected are common pathogens of humans and animals.

Antibacterial activity by disc diffusion assay

Disc diffusion assay was performed by the method of Arvind *et al.* (2015) with minor modifications [32]. Sterile discs of 6 mm diameter (HiMedia, India) were impregnated with CUD (25 µl), and the discs were allowed to dry overnight. CUD-impregnated discs were placed equidistantly on Muller Hinton Agar (MHA) plates inoculated with test bacterial strains. Ampicillin discs (10 µg) (HiMedia, India) were used as a positive control. MHA plates were incubated for 24 h and at 37°C and results were expressed as ZOI (zone of inhibition) in mm.

Antibacterial activity by well-diffusion assay

Agar well-diffusion assay was carried out following the methodology of Arvind *et al.* (2015) with slight modifications [32]. MHA plates were prepared and tested for sterility. Wells of 10 mm diameter were punched using a sterile cork-borer and sealed with sterile molten agar. Test bacterial strains (200 µl, 1:100 diluted) were poured on the MHA plates, spread with the help of a sterile L-spreader, and allowed to dry. CUDs (100µl) produced and procured for the study were poured into the wells to evaluate the antibacterial activity. Ampicillin (100µg/µl) was used as positive control. Plates were incubated for 37°C for 24 h and the results were recorded as ZOI in mm.

Microtiter plate assay (Broth dilution test)

Microtiter plate (MTP) assay was performed by the method of Sfeir *et al.* (2013) with minor modifications using sterile microwell plates with lid (APS, India) [33]. CUDs of Sahiwal, Tharparkar, and Vrindavani, produced in the LPT Division, two CUDs obtained from goshalas (Shahjanhpur and Kanerimath) and one commercial (C) were included. DW, ADW, and untreated culture were used as negative control while ampicillin (100 µg/µl) and chloramphenicol (35 µg/µl) was taken as positive control. CUDs (100 µl/ well) to be tested were poured column-wise into wells of a microtitre plate. Test bacterial strains (1: 100 diluted) were added row-wise (100 µl/well). All the tests were performed in duplicate. Sterile distilled water (DW) and alkaline distilled water (ADW, pH 9.4) were taken as negative controls, while ampicillin (100 µg/µl) and chloramphenicol (35 µg/µl) were taken as positive controls to compare the inhibitory activity of CUDs. Bacterial cultures without any treatment were marked as un-inoculated control. The plates were incubated at 37 °C for 24 h. After incubation, optical density (OD) was measured using a microplate reader (iMark, Biorad, Germany) at 595 nm. All the experiments were carried out in triplicates.

Minimum inhibitory concentration (MIC) of CUD as an antibacterial agent

MIC was determined following the methodology of Sfeir *et al.* (2013) with minor modifications [33]. MIC was determined by making a two-fold serial dilution of CUDs in sterile DW (undiluted to 1:32; column-wise). CUDs of Sahiwal, Tharparkar, and Vrindavani, produced in the LPT Division, two CUDs obtained from goshalas (Shahjanhpur and Kanerimath) and one commercial (C) were included. DW, ADW, and untreated inoculum were used as negative control while ampicillin (100 µg/µl) and chloramphenicol (35 µg/µl) were taken as positive control. Test bacterial strain (1:100 diluted) was inoculated to each dilution of CUDs (100 µl/well). Plates were incubated at 37 0C for 24 h. The optical density at 595 nm was measured using a microplate reader (iMark, Biorad, Germany).

Evaluation of CUD as bactericidal or bacteriostatic

The bacteriostatic or bactericidal potential of CUDs was tested by spreading 10 µl of treated and control samples from MTP assay on Mueller Hinton agar (MHA) plates (mixed with 190 µl of brain heart infusion broth). All the plates were incubated at 37ºC for 24h for development of visible growth (if any). The results were calculated as cfu/plate.

Antifungal activity by disc-diffusion assay

Antifungal activity of the CUDs was carried out by following the methodology of Arvind *et al.* (2015) with minor modifications [32]. Sterile discs of 6 mm (HiMedia, India) were impregnated with 25 µl of CUD and left overnight for drying. CUD discs were placed on Sabouraud dextrose agar (SDA) inoculated with 100 µl of test fungal strains. Antifungal disc (Clotrimazole 10 µg, HiMedia, India) was used as a positive control. The plates were incubated at room temperature (25±1ºC) for 3-5 days and the results were expressed as ZOI in mm.

Antifungal activity by tube method

The tube method was performed by adding 1 ml of test CUD to 1 ml of test fungal culture (*Candida albicans* ATCC 10231 and *Malassezia furfur* 12345) in Sabouraud dextrose broth (SDB). Untreated culture, ADW, and DW were used as negative control and the tubes were incubated at room temperature (25±1ºC) for 3-5 days. The results were determined by the presence or absence of growth in the form of turbidity.

Evaluation of CUD as mycostatic or mycocidal

The mycostatic or mycocidal potential of CUDs was evaluated by spreading 10 µl of treated and control samples from tubes on SDA plates. Plates were incubated at room temperature (25±1ºC) for 3-5 days and observed for the development of fungal colonies.

Evaluation of the antimicrobial activity of concentrated CUDs

CUDs were concentrated by freeze drying, re-dissolved in sterile DW (@ 25 mg/mL), and tested for antimicrobial activity by disc diffusion and MTP methods, as described in previous sections. Suitable positive (ampicillin) and negative controls (DW/ADW) were included. Interpretation was carried out by measuring ZOI or OD595.

Evaluation of the antimicrobial activity of CUDs in the vapor phase

To determine the vapor phase activity, the bacterial and fungal strains were inoculated on the surface of the MHA or SDA plates, respectively using a sterile L-spreader. CUDs (200 µl) were poured into the sterile cups attached to the lid of the Petri plate. The plates were incubated at 37 °C for 24 h for bacterial strains and at room temperature (25±1 °C) for 3-5 days for fungal strains and ZOI was measured in mm.

FT-IR analysis of CUD samples

Structural determination of different functional groups present in CUDs was carried out by FT-IR analysis (Nicolet 6700, Thermo, USA). CUDs were analyzed by placing the sample on the zinc selenide crystal of the attenuated total reflectance (ATR) assembly. The FT-IR spectra of extracts were recorded in the range of 4000-400 cm-1 using an FT-IR spectroscope with the aid of a DTGS-XT-KBr detector and XT-KBr beam splitter assembly for 32 scans at 4.0 cm-1 resolution [34].

Gas Chromatography-Mass Spectroscopy (GC-MS) analysis of CUD samples

The GC-MS analysis of the methanol fraction of the CUD was performed to identify the active constituents using an Agilent GC-MS mass instrument (Model 5975, Agilent Technologies, USA) equipped with a DB-5MS column (30 m × 250 µm × 0.25 µm). Helium was used as a carrier gas (1.0 mL min-1). The initial oven temperature was 500C with a hold time of 2 minutes. The temperature was programmed to rise by 8°C /min with a final temperature of 280ºC. The injection volume was 1 µL, the split ratio was 50:1, and the inlet temperature was 250ºC. The mass spectra were recorded with an electron energy of 70 eV over a range of 29-450 amu and an ion source temperature of 230ºC. Data handling was done using Chemstation software. Compounds in the extract were identified by matching their mass spectra with those of the National Institute of Standards and Technologies (NIST) USA, Library. Methanol was used as a solvent for making the dilutions [34].

RESULTS AND DISCUSSION

The pH of CUDs produced from fresh urine of different indigenous cow breeds ranged from 8.76 to 9.50 exhibiting their alkaline nature (Table 1). The pH of commercial CUD included in the study measured was 8.15 which was slightly less alkaline than CUDs produced, whereas the pH of CUDs procured from Goshalas of Shahjanpur and Kanerimath was measured

Table 1. pH of CUDs produced and procured.

Sl. No.	CUD	pH
1	S1	9.35
2	S2	9.43
3	S3	9.40
4	S4	9.42
5	S5	9.50
6	S6	9.34
7	T1	9.33
8	T2	9.46
9	T3	9.40
10	T4	9.43
11	T5	9.47
12	T6	9.42
13	V1	8.76
14	V2	8.95
15	C1	8.15
16	Gaushala, Shahjanpur	9.58
17	Gaushala, Kanerimath	9.30

(S= Sahiwal, T= Tharparkar, V= Vrindavani, C= Commercial).

to be 9.58 and 9.30, respectively, which were almost similar to CUDs produced in our laboratory. Sharma *et al.* (2017) measured the pH of CUD and found a similar range of pH (8.82) which is in agreement with the results of the present study [35].

Analysis of CUDs for various minerals revealed that CUDs did not contain calcium, phosphorus, zinc, copper, and iron. Cobalt was present at 0.25 ppm concentration in the CUDs.

CUDs did not exhibit antibacterial activity against any of the test bacterial strains in disc diffusion or well-diffusion assay as no ZOI was observed in any of the CUDs. Ampicillin discs produced variable ZOI from 8 mm to 26 mm depending on the genus of the test bacterium. Manjramkar *et al.* (2019) reported no activity in a laboratory-produced CUD, but commercial CUD produced ZOI of 8.58-18.55 mm and 3.0-15.21 mm against *S. aureus* and *E. coli*, respectively [21]. Sathasivam *et al.* (2010) produced CUD and observed

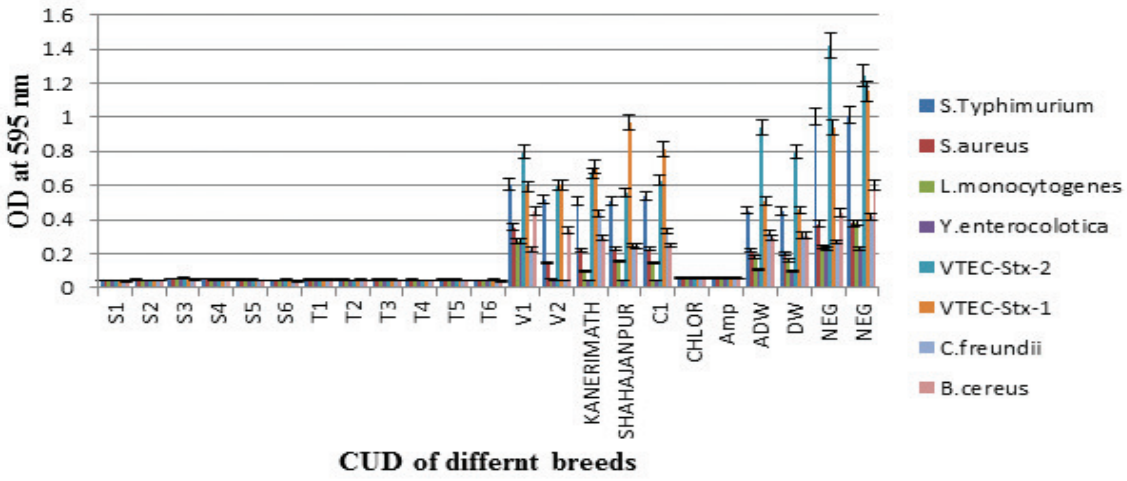


Fig. 1. Evaluation of antibacterial activity of CUDs against different bacterial strains by microtiter plate (MTP) assay.

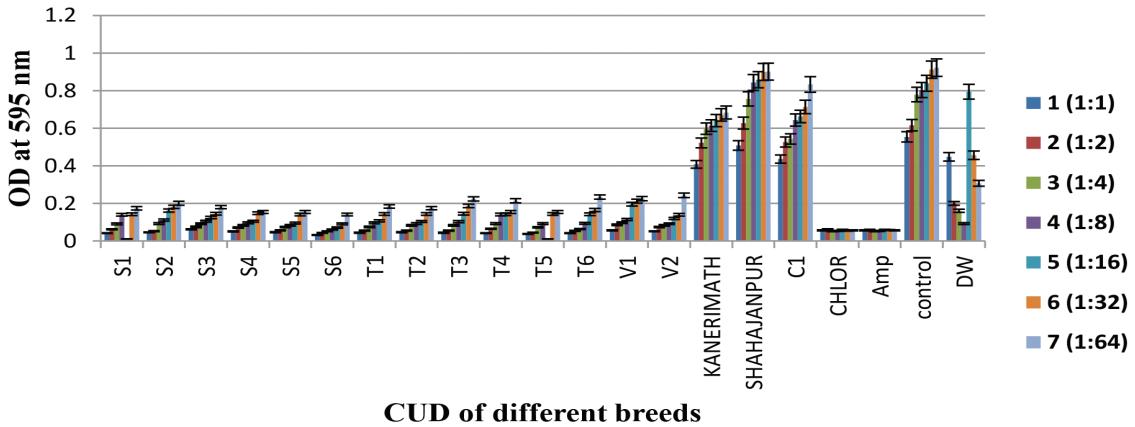


Fig. 2. Evaluation of minimal inhibitory concentration of CUDs against *Escherichia coli*.

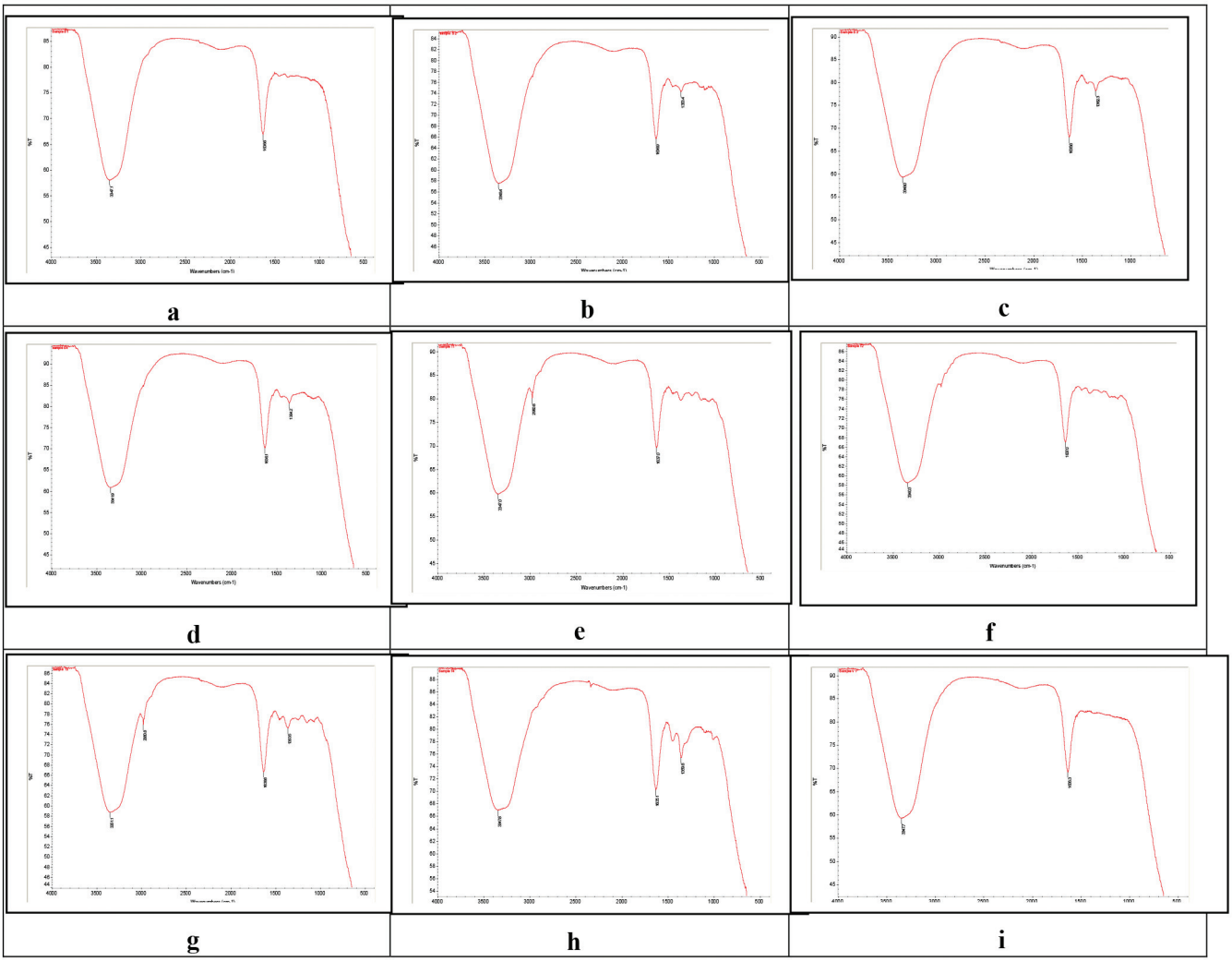
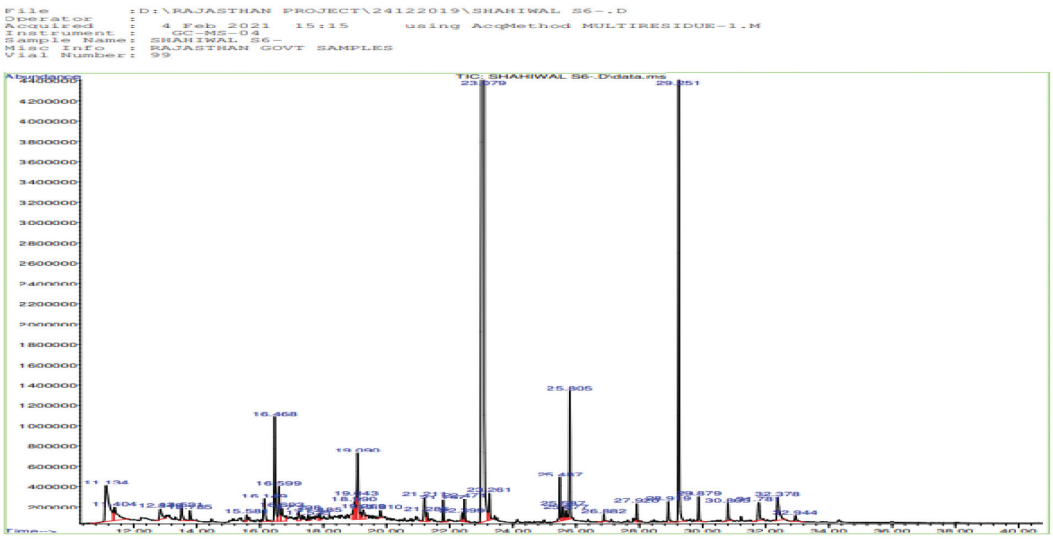


Fig. 3. FT-IR spectra of CUD samples (a) S1 CUD (b) S2 CUD (c) S3 CUD (d) S4 CUD, (e) T1 CUD (f) T2 CUD (g) T3 CUD, (h) T4 CUD and (i) V2 CUD.

the highest inhibition against *S. typhi* (15.4 mm) at 15 µl concentration [36]. Similarly, cow urine distillate was tested against some clinical pathogenic bacteria including *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Salmonella typhi*, and fungi including *Aspergillus niger* and *Aspergillus flavus* using disc diffusion method and found to be effective with variable ZOI [37, 38]. In another study, CUDs were found effective against *Staphylococcus aureus*, *E. coli*, *Pseudomonas*, *Bacillus subtilis*, *Proteus vulgaris*, *Streptococcus* spp., *Klebsiella pneumonia*, *Salmonella typhi*, and *Aspergillus* spp. strains by disc diffusion method [31]. In our study, ZOI could not be detected in any of the CUDs tested by disc (25 µl) or well (100 µl) diffusion methods which is in contrast to some of the previous studies that have reported the variable ZOI.

For disc diffusion assay which is performed mostly on Mueller Hinton agar plates, the active components of any antimicrobial should be able to diffuse into the medium. It is suspected that the active antimicrobial components of CUD were unable to penetrate the MHA plates and therefore could not produce any ZOI. The variation in the observed inhibition zone may also be attributed to the distillation process followed for the production of CUDs, the breed of cows used and their geographical location, feeding management, or the resistant status of the test strains of microbes used for the study.

Microtitre plate assay is commonly carried out to determine the MIC of antimicrobials and to determine the cidal or static nature of the antimicrobials, sub-culture is carried out onto a medium without antimicrobials. An antimicrobial should possess killing



4(a)

4(b)

4(c)

Fig. 4. GC-MS analysis of CUD samples (a) Sahiwal (S6); (b) Tharparkar (T6) and (c) Vrindavani (V2).

(cidal) potential rather than just inhibit the growth (static). The MTP assay exhibited good antibacterial activity in terms of no development of turbidity in CUD-treated samples and development of turbidity in untreated ones by visual observation. Lower absorbance represents a higher degree of inhibition of bacterial growth whereas higher absorbance represents a lesser degree of inhibition. The CUD produced from indigenous cow breeds (Sahiwal and Tharparkar) exhibited higher level of inhibition than CUD procured from crossbred cows (Vrindavani), CUDs procured from Goshalas (Shahjanpur and Kanerimath) and commercial CUD (C1) (Fig. 1). These results indicate that the CUD of indigenous breeds may be carrying some constituents responsible for their superior antimicrobial activity. Cow urine from indigenous cows has been reported to contain higher phenolic content which may contribute to its antibacterial action [21]. Additionally, photo-activation and distillation of cow urine might have enhanced the activity of CUD. Manjramkar *et al.*, 2019 also analyzed five commercial and one laboratory-produced CUD and reported a phenol coefficient of 0.03-0.04 against *S. aureus* and *E. coli* for commercial CUDs, while laboratory-produced CUD did not show a phenol coefficient [21].

To determine the MIC of the CUDs, serial two-fold dilutions were prepared. OD595 had a gradual increase with the increasing dilution of the tested CUDs. Undiluted CUDs (1:1) exhibited minimum OD595 indicating highest bacterial inhibition, while highest dilution (1:64) of CUDs tested exhibited maximum OD595 indicating lowest bacterial inhibition (Fig. 2). CUDs of Sahiwal, Tharparkar and Vrindavani exhibited significantly ($p<0.05$) higher inhibition up to 1:32 dilution whereas CUDs procured from goshalas (Kanerimath and Shahjanpur) and commercial CUD exhibited least inhibition, almost equivalent to DW and ADW control. Distilled water (DW) or alkaline distilled water (ADW) had no significant effect on bacterial growth, while ampicillin and chloramphenicol completely inhibited the growth of bacteria even at the highest dilution tested (1:64).

The CUDs produced exhibited much higher inhibition (lower OD595) even at higher dilution (1:32) in comparison to the commercially available CUD (1:2). This may be attributed to the higher phenolic content or other compounds yet to be identified. To evaluate CUD as bacteriostatic or bactericidal, 10 µl of treated and control samples from MTP assay after overnight incubation was plated on MHA plates. Plating of the treated and untreated samples resulted in no growth in the case of Gram-negative bacteria (C-173, C338, MTCC3231, MTCC2956, and ATCC23715), but few colonies appeared in case of Gram-positive bacteria (MTCC656, ATCC29213 and ATCC10876) indicating bactericidal effect of CUDs on Gram-negative bacteria and bacteriostatic effect on Gram-positive bacteria. No colonies appeared in ampicillin (positive) control, while numerous colonies appeared in negative control (untreated bacteria and bacteria treated with DW and ADW). However, the count of colonies in untreated control was much higher as compared to the count in Gram-positive bacteria treated with CUD.

In the antifungal assay, no ZOI was observed around any of the CUD-impregnated discs, whereas an inhibition zone was formed around the positive control disc ranging from 20-32 mm depending on the genus of the test fungus. In the tube method, growth of test fungal culture (*C. albicans*) in untreated, DW and ADW control was observed in terms of turbidity, while no growth occurred in tubes treated with CUDs of Sahiwal, Tharparkar, Kanerimath and Shahjanpur gaushala. To evaluate CUD as mycostatic or mycocidal, 10 µl of treated and control samples from tubes was spread on SDA plates. Plating of the treated and untreated samples on SDA plates resulted in no growth in any of the CUD-treated samples while spreading fungal growth appeared in negative controls (untreated, DW, and ADW treated), indicating mycocidal effect of CUDs on test fungus strain.

In the vapour phase activity assay, results demonstrated that CUD vapors have antibacterial and antifungal effects as ZOI was produced on the media surface by evaporated fumes of the CUDs. CUD vapors of the Tharparkar and Sahiwal breed produced relatively larger ZOI as compared to Vrindavani. Vapors of CUDs from goshalas (Kanerimath and Shahjanpur) and DW (negative control) failed to produce any ZOI. Cinnamon essential oil (CEO) used as positive control produced a wide ZOI (36 mm).

FT-IR spectra of CUDs exhibited bending vibrations at 1633.5 cm-1 due to N-H groups resembling N-H bonding in the structure of urea. Stretching vibrations at 3330.10 cm-1 are due to C-H group [34]. No significant difference in FT-IR spectra in CUDs produced from different breeds of indigenous cows was observed (Fig. 3). GC-MS analysis of methanol extract of CUD of Sahiwal, Tharparkar, and Vrindavani showed the presence of 105, 76, and 98 compounds, respectively. The GC-MS chromatograms of CUD of Sahiwal, Tharparkar, and Vrindavani are shown in

Fig. 4. GC-MS analysis revealed the presence of Dibutyl phthalate as a major compound in the concentration of 56.35%, 50.82%, and 59.65% in the CUD of Sahiwal, Tharparkar and Vrindavani, respectively. Diethyl fumarate was present in the concentration of 1.48, 5.02, and 0.55%; Bis (2-ethylhexyl) phthalate in 7.18, 1.58, and 6.37 %; and Quizalofop-p-ethyl in 1.32, 0.40 and 1.30 % in CUD of Sahiwal, Tharparkar and Vrindavani, respectively. GC-MS analysis revealed the presence of few unique secondary metabolites in Sahiwal and Tharparkar CUD including 3,4,5-Trimethylpyrazole in the concentration of 2.75 % and 14.43 %; 3-Aminophenol in the concentration of 0.56 and 1.68 %, respectively, which may have contributed to their superior antimicrobial activity compared to Vrindavani CUD [34].

In ancient Ayurvedic literature including *Charaka Samhita* and *Shushruta Samhita*, several medicinal properties of cow urine are described. Cow urine has been used as an appetizer, diuretic, nephroprotective agent, and insecticide. Cow urine has been prescribed to cure intestinal gas, acidity, cough, indigestion, stomachache, edema, colic, jaundice, anemia, diarrhea, gastric infection, piles, skin diseases including vitiligo, reversal of cardiac problems, kidney problems, and inflammation. Cow urine is reported as a universally available and easily digestible medicine and is claimed to make humans wiser [39]. Although various medicinal properties of cow urine have been reported in Ayurvedic texts but scientific evidence in support of these is grossly lacking [40]. Therefore, the present study was carried out to investigate the antimicrobial activity of CUD prepared from the fresh urine of indigenous cows and compare their activity.

The antimicrobial agents need to reach the site of infection in body fluids and tissues at the required concentrations to kill or inhibit the growth of the pathogen. Inadequate penetration of the infection site, degradation of antimicrobials by enzymes produced by pathogens, modification in the site of action of antimicrobials, and pumping out of the antimicrobials by pathogens using efflux pumps are some of the mechanisms related to the failure of antimicrobial therapy. An antimicrobial agent at a certain concentration may be bacteriostatic but may exhibit bactericidal effects at higher concentrations albeit against susceptible pathogens. These established principles apply to the efficacy of antibiotics and the same may apply to the antimicrobial activity of CUDs. Research work has not been conducted to establish the efficacy and mechanism of antimicrobial action of CUDs. We have initiated to establish that CUD is active against human pathogens *viz., E. coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Staphylococcus aureus, Bacillus subtilis, Streptococcus pyogenes*, etc. Yet, the exact mode of action of CUD against these organisms is not clear. Enough data on high-performance liquid chromatography of CUDs is currently not available therefore there is a considerable gap regarding the active molecules, their pharmacokinetics, and pharmacodynamics properties. Without such data, it is difficult to establish the mode of action of antimicrobial activity exhibited by CUDs.

Cow urine has been known to be bactericidal from time immemorial. Even though cow urine has been revered from ancient times, there are a lot of hurdles that research on CUDs needs to clear before considering it to enter into mainstream. The current research focussed only on the *in vitro* effectiveness of CUDs against a few important microbial pathogens. Still, scant laboratory data has been generated on the pharmacologically active molecules and possible mechanisms of action. Cow urine and CUDs have been tested for various disease conditions in animals, but that is far from being qualified to be called controlled animal testing. Despite a few patents granted for CUD-based products, there is a formidable research process to cover before it can be declared safe for human use.

In the last four decades, AMR has led to the generation of "superbugs" *i.e.* super-resistant strains with enhanced virulence and transmissibility. Since the development of resistance is a natural phenomenon, the emergence of antibiotic resistance cannot be stopped but its onset can be delayed. AMR is not restricted to human beings but is also continuously spreading to economically important livestock. Although the development of a new antimicrobial agent is always the first choice, alternative antimicrobials can be effectively used to reduce the dependence on antimicrobials which in turn can delay the onset of AMR. Alternative antimicrobials can be extremely useful in the current situation where no new antibiotic candidate is in the discovery pipeline.

Antimicrobial resistance is a global threat therefore multi-pronged approaches are to be researched to tackle it. These include natural products, new compounds with previously unknown mechanisms, mutated bacterial toxins, bacteriophage therapy, bacteriocins, antimicrobial peptides, and nanoparticles. If some of these products can successfully be translated into therapeutic regimes, the bacteriological apocalypse

may be averted. The results of this study indicate and confirm that CUDs carry antimicrobial potential as mentioned in the ancient literature and reported earlier by various researchers. However, further work is required to validate these results *in vivo* using a suitable experimental animal model system, to identify the compounds responsible for the antimicrobial activity, and to establish the mechanism of its action.

CONCLUSION

The urine distillates prepared from fresh urine of indigenous cows (Sahiwal and Tharparkar) demonstrated broad-spectrum antimicrobial activity. The distillates were cidal to Gram-negative but static to Gram-positive bacteria tested under the study. The activity was also observed in the vapour phase. GC-MS analysis identified a few compounds in the distillates of Sahiwal and Tharparkar urine which may be responsible for this antimicrobial activity. The compounds identified in distillates can be utilized as alternative antimicrobials against resistant microbes, however, further work needs to be carried out on this aspect.

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