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Evaluation of *in vitro* Anti-Microbial Activity of Goat Urine Peptides

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ABSTRACT

Indiscriminate uses of antibiotics have caused microbial resistance and also lead to many side effects. To overcome from such situation plants and animal materials are widely used the treating various ailments having antimicrobial properties. In Ayurveda, goat urine has been used to improve general health of an individual. Therefore, present study was undertaken to study *in vitro* antibacterial potential of urinary peptides of goat against *S. aureus* and *E. coli*. The method employed extraction of urinary peptides from goat urine and subsequently antibacterial activity of extracted urinary peptides was studied by radial diffusion assay technique and microtiter broth dilution method. The results showed good antibacterial activity of goat urinary cationic antimicrobial proteins against test bacterial strains by exhibiting significant zone of inhibition. Thus it can be concluded that cationic urinary peptides of goat possess good inhibitory activities against bacterial strains and can be used to control infectious diseases.

Keywords: Antimicrobial activity, urinary peptide, goat

Antibiotics are widely used in treatment of various microbial diseases (Daniel, 2005). However the major hindrance is that the bacteria are having genetic ability to transmit and acquire resistance towards the drugs (Cohen, 1992). Thus, the injudicious use of antibiotics not only enhancing drug resistance but also causing adverse effects on hosts (Ahmad *et al.,* 2005). To combat the problems of drug resistance and its adverse effect on hosts, many natural products having antibacterial properties are being explored. In mammals various potential antibacterial proteins are naturally synthesized and reported to be released in their secretions like lysozyme in tears and saliva. In ayurveda, products of goat like urine, dung, milk, ghee and curd are widely used in number of medicinal formulations used in curing various diseases like skin affections, kidney problems, epilepsy, anemia, constipation, respiratory disease etc. Based on the literature search, it was hypothesized that medicinal properties of goat urine may be due to presence of some of the secreted antimicrobial peptides in the urine of goat. Thus the present investigation was designed to extract urinary proteins of goat and explored for their antibacterial potential against *S. aureus and E. coli* bacterial strains.

MATERIALS AND METHODS

Animals and urine collection

Goats used for the study were belonged to goat shed

of Department of Veterinary Physiology, College of Veterinary Science, Mathura. Around 30-80 ml of urine samples were collected in morning hour from ten clinical healthy female goats (age >1 year) each in sterile wide mouth container. Immediately after the collection samples were transported to the laboratory at 4°C. Then urine samples were analyzed for physio-chemical parameters of the urine (pH, specific gravity, total protein, urea, uric acid, creatinine, glucose, ketone bodies) by using Reagent Dip strips for urinalysis (SPAN-ARKRAY Healthcare, India). The urine samples with normal biochemical parameters were pooled together and protease inhibitor phenyl methyl sulfonyl fluoride (PMSF, 0.01%) was added to inhibit the degradation of proteins. The pooled urine samples were subjected to filtration through 0.2µ membrane filter for removing any insoluble materials and bacterial contamination present in urine samples.

Sample preparation

The freshly collected pooled urine was centrifuged at 6000 rpm for 30 minutes to remove insoluble materials. Subsequently, the protein present in urine was concentrated by centrifugation using 10 kDa membrane filter (Amicon Ultra-4 Centrifugal Filter Unit with the Ultracel-10 Membrane, MILLIPORE) by centrifugation at 4000g for 20 minutes at 4°C. By this approach, one liter of urine was concentrated to 100 ml and subsequently, samples were stored at 4°C till further use.

Extraction of peptides

The protein present in 100 ml of concentrated urine (Dia-filtered urine) was extracted using weak Cation Exchange beads (Macro Prep®CM Resin, BIO-RAD India) as per the method described by Valore *et al.* (1998) with slight modification. For that 100 ml of concentrated urine was mixed with 100 µl of CM-Macro prep (50% Slurry) and continuously stirred at room temperature for 2 hours at 4°C and then the beads were sedimented by centrifugation at 200 g. The unbound peptides/proteins were removed by washing the beads with approximately two bed volume of 25 mM ammonium acetate (pH 7.5) by centrifugation at 200 g for 10 minutes. This step of washing was repeated for 5 times for ensuring the complete removal of unbound peptides (i.e. anionic and neutral peptides). The peptides (cationic) bound to CM-Macro prep were extracted by adding two volumes of 5% acetic acid and incubating for at least 20 min at 4 °C and then beads were separated by centrifugation at 200 g. This step was repeated twice for complete recovery of bound cationic peptides. All the fraction containing bound and unbound peptides were concentrated by evaporating the solvents by vacuum drying using Speed Vac™ Concentrator (Savant™ Thermo-Fisher Scientific, India). Afterwards all the dried samples were dissolved in autoclaved triple distilled water.

Protein assay

After extraction, protein concentration was determined by Bicinchoninic acid assay method (BCA assay) using Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific™).

Evaluation of antimicrobial activity

The antimicrobial activity of extracted fractions of urinary peptides was carried out using radial diffusion assay (RDA) and micro titer broth dilution method.

Radial diffusion assay

The evaluation of antimicrobial activity of extracted urinary protein fractions was performed using radial diffusion assay as described by Lehrer *et al.* (1991) with slight modifications. The single pure colony of *Escherichia coli* ATCC 25922 and *Staphyloccus aureus* ATCC 29213 strains were grown in Muller Hinton Broth (MHB) by overnight incubation at 37°C. After rapidly dispersing the bacteria with a laboratory vortex mixer, 20 ml agar was poured into a round petri dish (Hi-Media) on a perfectly level platform, to form a uniform layer of approximately 1 mm deep. The plates were allowed to solidify and a 3 mm diameter gel punch was used to make 6 evenly spaced wells. Each well was loaded with 10 µl of extracted and dissolved urinary proteins/peptides. The plates were incubated for 3 h at 37 °C and then overlayed with 10 ml of sterile agar that was maintained in fluid phase at 42°C. The overlayed agar consisted of a double-strength (6% w/v) solution of MHB and 1% w/v agarose. After incubation for 18-24 h at 37 °C, the diameter of the clear zone surrounding the wells was measured with the ruler scale.

Microtiter broth dilution method

Minimal inhibitory concentration (MIC) value was determined by broth micro dilution method (Wiegand *et al.,* 2008) for evaluation and quantification of susceptibilities of bacteria to urinary peptides of goats. This method was used to determine the lowest concentration of urinary antimicrobial peptides that inhibits the growth of the bacterium under defined test conditions. MHB (100 µL) was dispensed into 12 wells in a row (A1 to F1) of sterile micro-titre plate. Then 100µL of urinary antimicrobial peptide solution was added into the first well containing broth and mixed by sucking up and down for 5-8 times using pipette. Thereafter 100 µL of mixture from this well is transferred to second well mixed by sucking up and down and procedure was repeated up to the 10^{th} well and 100 µL solution was discarded from 10^{th} well. Then 5 µL of mid log phase of growing culture as described earlier containing 5×10^7 cfu/ml of *E. coli* and *S. aureus* were transferred into wells of column 1 to 11. The 12^{th} column serves as background control. Plates were incubated at 37°C and the change in optical density was measured by using ELISA reader at 620 nm.

RESULTS AND DISCUSSION

The results of antimicrobial activity of both cationic and anionic fractions of urinary peptides by radial diffusion assay against the *E. coli* and *S. aureus* has been depicted in (Table 1, and Fig. 1 & 2).

Table 1: Zone of inhibition in RDA against different fractions of urinary peptides of goat

Microorganism	AF1	AF2	AF3	AF4	AF5	CF
S. aureus	NZO	NZO	NZO	NZO	NZO	23mm
E. coli	NZO	NZO	NZO	NZO	NZO	26mm

Table 2: Minimum inhibitory concentration in micro-titer broth dilution against different fractions of urinary peptide of goat

Microorganism	Minimum inhibitory concentration µg/µl
E. coli	0.039
S. aureus	0.0199

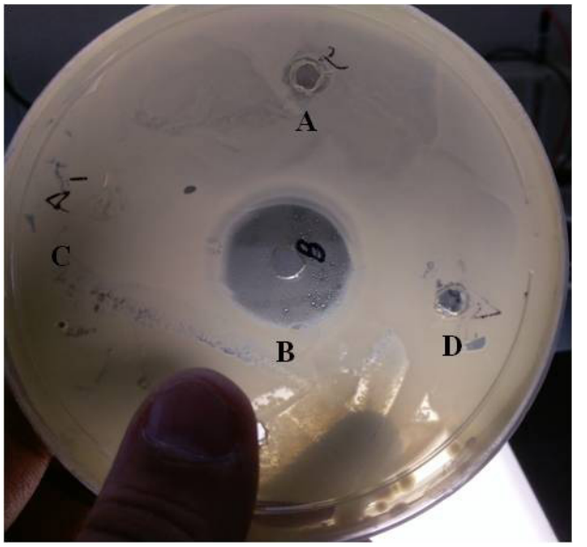

Fig. 1: Radial diffusion assay showing zone of inhibition against *E.coli.* Wells: A & C- Contains Anionic peptide; B- Contains Cationic peptide; D- Contains negative control

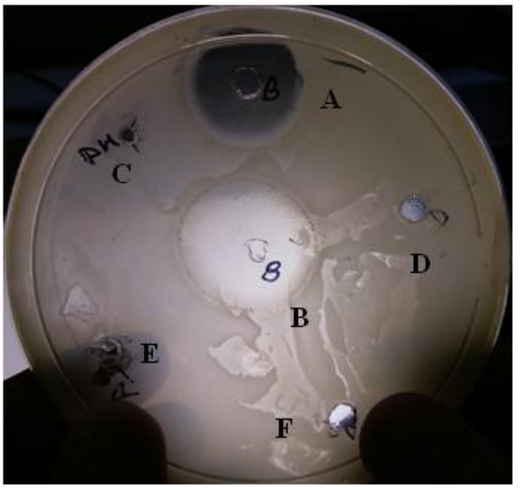

Fig. 2: Radial diffusion assay showing zone of inhibition against *S aureus*. Wells: A & B- Contains cationic peptide; C, E & F- Contains anionic peptide; D- Contains negative control

The results of RDA revealed significant antimicrobial activity in fraction containing cationic peptides against *S. aureus* and *E. coli* which showed 23 mm and 26 mm of zone of inhibition, respectively. While No antimicrobial activity was observed in all other fractions containing neutral/anionic peptides as evident by absence of zone of

inhibition. The result of determination of MIC value of cationic urinary peptides of goats against *E. coli* and *S. aureus* revealed was observed 0.039 µg/µl and 0.0199 µg/µl, respectively. The results of the RDA clearly indicated that the cationic peptides exhibited significant inhibitory potential against *S. aureus* and *E. coli*.

The results of RDA revealed significant antimicrobial activity in cationic peptide fractions against *S. aureus* and *E. coli.* The result of RDA was compared with earlier reports of RDA carried out on urine or urine distillate of other animals. Edwin *et al.* (2008) reported significant anti-microbial activity of cow urine and its distillate and observed comparable zone of inhibition against *S. aureus* (22 mm) and *E. coli* (23 mm). Similarly Shah *et al.* (2010) determined the antimicrobial effect of photo-activated and fresh urine of cow and revealed significant zone of inhibition against *S. aureus* (17 and 18 mm) and *E. coli* (13 and 16 mm), respectively which were comparable with the effect of standard antibiotic streptomycin (16 and 26 mm), respectively. However Sumia *et al.* (2016) studied the anti-microbial activity of fresh and concentrated sheep urine and revealed no zone of inhibition against different bacterial strains using sheep urine. Sathasivam *et al.*(2010) reported antimicrobial activity of urine and demonstrated that the bactericidal effect of urine may be due to its specific acidic pH or may be due to presence of amino acid / urinary peptides which increases bacterial and cell surface hydrophobicity (Badadani *et al.,* 2007). The significant antimicrobial effect of goat urine in present study indicated that the anti-microbial activity of urine is not due to the pH effect of urine because pH of goats urine used was alkaline (pH 9.0) that cannot inhibit the bacterial growth. Moreover results of present study also depicted that the anionic fractions did not reveal zone of inhibition against studied bacterial growth. Thus the findings of the present study indicating that the cationic peptides present in the goat urine contributes in the antimicrobial activity of urine.

Since cationic fraction showed very high susceptibility to studied bacterial strains, its minimum inhibitory concentrations (MICs) was determined by broth micro-dilution method which is reference method for anti-microbial susceptibility testing and widely used to establish minimum concentration required to inhibit the growth of microorganism. The MIC value for cationic fraction of urine obtained was 0.0199 µg/µl against *S. aureus* and 0.039 µg/µl against *E. coli* that significantly inhibited the growth of the bacterium under defined test conditions. However Upadhyay *et al.* (2009) estimated the MIC values as 0.25 and 0.125 µl/ml in photo-activated cow urine. The results of MIC values for cationic peptide of urine observed in this test could not be compared due to scarcity of literature describing the MIC values for cationic peptides of urine.

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