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Original Article

Antimicrobial Effect of Cow Urine and Goat Urine against common Dental Caries Pathogens: An *in vitro* Comparative Study

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ABSTRACT

Background- The emergence of resistant bacterial strains due to overuse of antibiotics has led to increase in use of natural animal products with known antimicrobial properties as therapeutic agents. **Objectives-** To assess the antimicrobial potential and Minimum Inhibitory Concentration (MIC) of cow urine and goat urine against common dental caries pathogens. **Materials and method:** Distillates prepared from cow urine and goat urine samples were assessed for its antimicrobial activity and minimal inhibitory concentration (MIC) against *Streptococcus mutans and Lactobacillus acidophilus* using agar well diffusion method. Statistical analysis was performed with One Way Analysis of Variance (ANOVA) using SPSS (Statistical Package for Social Sciences) version 19. **Results:** Both urine samples showed potent antimicrobial activity with MIC values ranging from 5-10µg/ml against dental caries pathogens. In particular, goat urine sample showed least MIC values of 5µg/ml against *S. mutans, L. acidophilus*. A statistically significant ($p<0.05$) zone of inhibition was observed for all samples against *S. mutans* at highest concentration (50µg/ml). Goat urine resisted the micro-organisms effectively compared to cow urine. **Conclusion-** Samples of cow urine and goat urine were found to be effective with therapeutic potential against dental caries pathogens. On comparison, goat urine was found to be more potent.

Key words: agar, cow urine, goat urine, *Streptococcus mutans*, *Lactobacillus acidophilus*.

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INTRODUCTION

Oral diseases continue to be a major health problem worldwide[1]. Dental caries is among the most important global oral health problems. Oral health is integral to general well-being and relates to the quality of life that extends beyond the functions of the craniofacial complex. The essential process of dental caries occurrence involves demineralization of tooth enamel, and likely also of root surfaces, by high concentrations of organic acids produced by bacteria in dental plaque from dietary carbohydrates.

Dental caries development is considered to involve a triad of indispensable factors: bacteria (dental plaque), carbohydrates (the diet), and susceptible teeth (the host)[2]. The acidogenic plaque bacteria, especially *Streptococci Mutans* and *Lactobacillus Acidophilus*, are associated with stages in the development of dental caries[3, 4]. Many studies have shown frequent association between the presence of *S. mutans* & *lactobacilli* with the prevalence of dental caries.[2, 5-9] Plaque from individuals with periodontal disease has been associated with a wide variety of micro-organisms.

Even though pharmacological industries have produced a number of new antibiotics in the last three decades, resistance to these drugs by microorganisms has increased[10, 11].The ultimate goal is to offer appropriate and efficient antimicrobial drugs to the patient. Focus on animal product research has increased all over the world and large body of evidence has collected to show immense potential of animal products used for their

therapeutic abilities against microbes. According to various health organisations, animal products would be an appropriate source to obtain a variety of drugs, therefore such products should be investigated to understand their properties, safety and efficacy for a search of new potent antimicrobial compounds.

Cow, *Bos indicus* is called as the Mother of all according to Veda's[12] and is compared to the nectar. Cow urine has a unique place in Ayurveda and has been described in 'Sushrita Sumhita' and Ashtanga Sangraha' to be the most effective substance secretion of animal origin with innumerable therapeutic values. Several medicinal properties of cow's urine have been mentioned and are known to cause immunomodulatory[13], hypoglycemic [14] and cardio-respiratory effects[15]. It is also used with herbs to cure diseases like fever, epilepsy, anemia, abdominal pain, constipation, etc. by the traditional healers[12]. In India, drinking of cow urine has been practiced for thousands of years. Panchagavya is a term used in Ayurveda to describe five important substances obtained from cow namely Urine, Dung, Milk, Ghee and curd. Urine therapy was not only used in India, but for several Centuries in many parts of the Globe. Recently the cow urine has been granted U.S. Patents (No. 6,896,907 and 6,410,059) for its medicinal properties, particularly for its use along with antibiotics for the control of bacterial infection[12]. Still, there is a great paucity of information on antimicrobial property of cow urine, especially those with oral manifestations.

Goats (*Capra aegagrus hircus*) are one of the oldest domesticated species, and have been used for their milk, meat, hair, and skins over much of the world. To the best of our knowledge, there is no literature available about the antimicrobial effect of goat urine against the microorganisms to be tested in the present study.

METHODOLOGY

The study protocol was reviewed and approved by the concerned Ethical Committee.

Procurement of urine sample

Cow urine and goat urine were collected from a local dairy farm. Animals (age 5-10 years) were maintained on the normal feed and water, and fed *ad-libitum*. Pregnant animals and those with any other infections were excluded from urine collection. The urine was collected in sterile bottles in the morning (between 6.00 and 8.00 am) kept in ice box, after discarding the first flow, were confirmed in the Department of Pharmacgnosy, Pacific College of Pharmacy, Udaipur. In the laboratory, both urine samples were distilled at 100°C using distillation apparatus. The distilled urine samples were acidified by lowering the pH below 2.0 with the addition of 85% orthophosphoric acid. They were again distilled at 100°C using a distillation apparatus to remove ammonia. The distillate were later stored in sterile glass flask at refrigerator (4°C) for further procedures.

Test Microorganisms

Two dental caries causing bacteria, *Streptococcus mutans* (MTCC*497) and *Lactobacillus acidophilus* (MTCC*447) were procured from Microbial Type Culture Collection, IMTECH, Chandigarh. Bacteria were grown in brain heart infusion broth attained from HiMedia Laboratory Pvt. Ltd., Bombay, India supplemented with yeast extract (0.5%), hemin (5µg/ml), menadione (1µg/ml) and incubated anaerobically at 37°C. Identification of all strains was confirmed by standard biochemical and straining methods.

Screening for Antimicrobial Activity

The distillates of cow urine and goat urinewere used for the antimicrobial screening using the agar well diffusion method. The media was punched with 7mm diameter wells and were filled with various concentrations of the samples at 2µg/ml, 5µg/ml, 10µg/ml, 25µg/ml and 50µg/ml. The plates were then incubated at 37ºC for 24 hours. After incubation, zone of growth inhibition for each extract was measured in millimetres using vernier callipers. Each sample was tested five times.

Determination of Minimum Inhibitory Concentration (MIC)

MIC is defined as the lowest concentration of a compound/extract/drug that completely inhibits the growth of the microorganism in 24 hours[16]. For MIC, 9 dilutions of each sample were done with brain heart infusion (BHI) broth microdilution assay. In the initial tube 20 microliter of sample was added into the 380 microliter of BHI broth. For dilutions, 200 microliter of BHI broth was added into the next 9 tubes separately. Then from the initial tube 200 microliter was transferred to the first tube containing 200 microliter of BHI broth. This was considered as 10^{-1} dilution. From 10^{-1} diluted tube, 200 microliter was transferred to second tube to make 10^{-2} dilution. The serial dilution was repeated up to 10^{-9} dilution for each sample. From the maintained stock cultures of required organisms, 5 microliter was taken and added into 2ml of BHI (brain heart infusion) broth. In each serially diluted tube, 200 microliter of above culture suspension was added. The tubes were incubated at 37°C for 24 hours and observed for turbidity.

Statistical analysis

The data obtained will be analysed using SPSS (Statistical Package for Social Sciences) version 19 (SPSS Inc., Chicago, Illinois, USA). Descriptive statistics (Mean value and SD) were calculated and comparisons of mean zone of inhibition between the samples and at different concentrations of both urine samples were performed using One Way Analysis of Variance (ANOVA). Level of significance and confidence level were set at 5% and 95% respectively.

RESULTS

(Table 1): Variation in antimicrobial activity of cow urine and goat urine was seen against two major dental caries pathogens. Cow urine was found to be effective against *S.*

mutans at the lower concentration (10µg/ml). Goat urine was found to be more effective and showed a statistically significant mean zone of inhibition (8.3±0.44) at a lower concentration of 5µg/ml (p=0.00). *L.acidophilus* showed resistance against cow urine at 5 µg/ml but showed mean zone of inhibition at 10µg/ml compared to goat urine which was highly effective at 5µg/ml with a mean zone of inhibition of 8±0.35

MIC values of all the samples against test pathogens mostly ranged between 5-10µg/ml (Table 2): Goat urine was found to be more sensitive against all bacterium compared to cow urine. *S. mutans* and *L. acidophilus* were found to more sensitive to goat urine samples compared to cow urine with Minimum Inhibitory Concentration of 5µg/ml. The effectiveness of cow urine was lesser compared to goat urine samples with higher values of MIC.

(Table 3): Biochemical analysis of both urine samples was done and the constituents were detected. The components were more or less similar in both the urine samples with the exception of *p*-Ethylphenylsulphuric acid which is found particularly in goat urine samples.

Table 1: Mean zone of inhibition (mm) of all samples of cow urine and goat urine on various dental caries and periodontal pathogens.

	CONCENTRATIONS (MEAN±S.D)					**P value**
S. mutans	**50** µg/ml	**25** µg/ml	**10** µg/ml	**5** µg/ml	**2**µg/ml	
Cow Urine	12.7±0.45	10±0.35	8.2±0.27	R	R	0.00*
Goat Urine	15±0.35	12.4±0.42	11.3±0.45	8.3±0.44	R	0.00*
L.acidophilus						
Cow Urine	9.3±0.44	8.3±0.44	7.4±0.41	R	R	0.00*
Goat Urine	11.4±0.41	10.3±0.44	8±0.35	8±0.35	R	0.00*

Test applied – ANOVA. *P≤0.05 statistically significant, R= Resistant

Table 2: Minimum Inhibitory Concentration (MIC) of cow urine and goat urine samples against dental caries pathogens on specific media for each microorganism.

Sample	***S mutans* (µg/ml)**	***L acidophilus* (µg/ml)**
Cow urine	10	10
Goat urine	5	5

Table 3: Biochemical analysis of both urine samples.

Chemical Constituent	***Cow urine***	***Goat urine***
Urea nitrogen	+	+
Ammonia nitrogen	+	+
Total nitrogen	+	+
phenols	+	+
***p*-Ethylphenylsulphuric acid**	-	+
Allantoin	+	-
Calcium	+	+
Chloride	+	-
Coproporphyrin	-	+
Creatinine	+	+
Magnesium	-	+
Potassium	+	+
Sodium	+	+
Sulphate	+	+
Uric acid	+	+
Uroporphyrin	+	+
Glucose	Nil	Nil
Protein	Nil	Nil
Haemoglobin	Nil	Nil

DISCUSSION

Antibiotics are being widely used these days for conservative treatment in various microbial infections and diseases.[17] Taking in account the enormous quantity of antibiotics used, the population using antibiotics should have been free from infectious disease. But, the fact is that the problems of infectious diseases are increasing day-by-day. Some of the major hindrances are that bacteria have genetic ability to transmit and acquire resistance towards the drugs[18] and there are also adverse effects of drugs on the host.[19] The increasing prevalence of antibiotic resistance in infectious bacteria, ultimately increasing prevalence of infectious diseases has raised the demand for the scientific community to search for new anti-bacterial components.[20,21]Therefore to combat such problems, the focus is now slowly but steadily shifting towards natural and herbal products. Natural sources are the best way to find new and noble anti-bacterial substances that can help to resolve this problem to some extent. The nature is an almost infinite resource for drug development and discovery. It has endowed mankind with a complete repository of remedies to cure all ailments of mankind, as it has always been a first rate drug store with enormous range of plants, micro-organisms and animals.[22]

The literature on cow urine and goat urine has always focussed on prevention of disease and maintaining the health and treatment of diseases. In India specially, as well as various other parts of the world, the medicinal properties of cow urine are well recognized and used since centuries to cure various ailments of the human body. In our study, we included goat urine also since it has also been identified as a potential anti-microbial agent and of use in human population. According to our knowledge, this is the first study that evaluates the effect of goat urine on oral pathogens. Also, there is scarce knowledge about the antimicrobial effect of cow urine on oral pathogens. Therefore, the present study was designed to evaluate the anti-microbial effect of cow urine and goat urine against dental caries and periodontal pathogens.

Antimicrobial efficacy is usually determined by examining minimum inhibitory concentration, bactericidal effects and other test that commonly utilize various microbial culture techniques.[23,24,25] In the present study cultural method employed was agar well diffusion method which offered several advantages such as selective quantification of microorganisms[26] but are laborious and only enumerate bacteria that can grow on agar.

Results originated from the assessment of the antimicrobial activity of the samples against *S. mutans* in the present study showed that at lower concentration (5µg/ml), goat urine was effective against this facultative anaerobic, gram-positive bacterium mainly due to the presence of high quantity of nitrogen and phenols in the urine.[27]Similar results were obtained among the two urine samples when assessing the antimicrobial effect on other gram positive bacterium *L. acidophilus*, with the bacteria showing inhibiting activity at lower concentration in goat urine sample compared to the cow urine sample. The results obtained showed that both the urine samples were capable of inhibiting the dental caries causing bacteria at different concentrations thus proving their potential to be an excellent alternative as a natural alternative to antibiotics. They are capable of inhibiting the bacteria that causes initiation of dental caries i.e. *S. mutans* as well as the one which causes progression i.e. *L. acidophilus*. Meanwhile on comparison with each other, the goat urine was found to be a stronger alternative as compared to cow urine with a lower mean zone of inhibition for both the bacteria.

The findings related to cow urine are in conjugation with the findings by Jarald E et al. (2008) who evaluated the antioxidant and antimicrobial activities of cow urine indicating positive correlation and acclaiming the traditional values of cow urine.[28] Sathasivam A et al. (2010) also conducted a study to evaluate the antimicrobial activities of cow urine distillate against some clinical pathogens and found that cow urine distillate has antibacterial activities and the inhibitory activity can be used in the control of bacteria of various origins.[29]

In microbiology, the MIC is the lowest concentration of an antimicrobial that inhibits the visible growth of a microorganism after overnight incubation[30]. *S. mutans* and *L. acidophilus* were found to be sensitive in goat urine having MIC value of 5µg/ml. This suggests the minimum concentration of goat urine required to inhibit the growth of dental caries pathogens. The findings for goat urine samples were stronger compared to the findings obtained with cow urine whose MIC was at a higher concentration. Though, cow urine was found to be an excellent substitute to the conventional approach of antibiotics against these bacteria which is in agreement with the findings of Raad et al. (2013)[31], the results of goat urine provides a new insight to look upon as it hasn't been considered before for use in oral health care products.

Conventionally, in-vitro or laboratory research studies have good internal validity but poor external validity which means that the results obtained are only applicable to similar samples of the study. In other words, the results may not transfer to the clinical behaviour of the material. On the other hand, clinical studies have good external validity because they are tested on samples/subjects that are closely related to the clinical condition and most often representative of all individuals with the condition.[32] In future in vivo clinical studies are warranted to confirm in vitro results and for the assessment of safety and efficacy of all the samples. Research should also be directed towards the incorporation of these extracts into dental products such as toothpaste and mouth rinse.

Today, most pathogenic organisms are becoming resistant to antibiotics.[33] To overcome this alarming problem, the discovery of novel active compounds against new targets is a matter of urgency. For a long time, animal products have been an important source of natural products for human health. The antimicrobial properties of these products have been investigated by a number of studies worldwide and many of them have been used as

therapeutic alternatives because of their antimicrobial properties.

Goat urine and cow urine have the potential to become promising natural antimicrobial agent in pharmaceutical industry for controlling the oral pathogenic bacteria. However, if these animal products are to be used for medicinal purposes, issues of safety and toxicity will always need to be considered. Also, the compliance and the rate of acceptance of these products need to be taken care of.

CONCLUSION

Both the tested animal products- cow urine and goat urine samples were found to be sensitive against *S. Mutans* and *L. acidophilus* respectively. On comparison, it was observed that goat urine sample emerged as stronger agent exhibiting antibacterial activity. Need of the hour is to execute more and more screening of natural products to set a primary platform for further biochemical, pharmacological and in vivo studies that may open the possibilities of finding new clinically effective antibacterial compounds against dental caries pathogens.

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