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Full Length Research Paper

Antimicrobial activity of photo-activated cow urine against certain pathogenic bacterial strains

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In the present investigation, binary combination of photo activated cow urine was determined against seven bacterial strains. Photoactivated cow urine showed MIC value of 0.25 µl/ml against *Staphylococcus aureus* (ATCC 25923), *Bacillus cereus* (ATCC 11778), *Lactobacillus acidophilus* (ATCC 53103 and *Micrococcus luteus* (ATCC 9341), while it had 0.125 µl/ml against *Escherichia coli* (ATCC 25922). Binary combinations of cow urine with Neem and Bavchi oil showed synergistic effect as the MIC value obtained was between 0.125 - 0.25 µl/ml. Similarly, photoactivated cow urine showed least MBC value of 0.25 - 1.0 µl/ml. Furthermore, growth inhibition zone diameters obtained in the presence of photoactivated cow urine and its binary combinations were found much larger than antibiotic drugs. Neem oil showed highest inhibition zone diameter of 45 mm against *Streptococcus pneumoniae*. Neem oil and cow urine showed 33 - 35 mm inhibition zone diameter against *B. cereus, L. acidophilus, Micrococcus luteus, Klebsiella pneumoniae and S. pneumoniae,* while Bawchi oil and cow urine combination showed a higher inhibition zone diameter of 41 mm against *K. pneumoniae.* Photoactivated cow urine showed 32 - 36 mm inhibition zone diameter homogeneously against all bacterial strains. It proved very high antimicrobial susceptibility of cow urine and essential oils.

Key words: Antimicrobial activity, cow urine and essential oil.

INTRODUCTION

For the control of microbial infections and diseases, various synthetic drugs and chemical formulations have been used. But due to their indiscriminate use, microbes have developed wide resistance against these synthetic drugs such as broad-spectrum antibiotics. This resistance was developed after induction of new enzyme system in microbes, which not only simplify drugs but also enhanced drug threshold level in microbes. Therefore, to combat the problem of microbial infection and drug resistance, new alternative to synthetic drugs have been explored, though antimicrobial activities of so many natural pesticides have also been explored. But there is no report available on antimicrobial activity of cow urine. In spite of the fact that cow urine has great pharmacological importance and has great aesthetic and medicinal value, its utility has only been mentioned in the holy texts of Indian literature. Cow urine has certain volatile and non-volatile components, which might have very high antimicrobial activity (Shaw et al., 2007). After photo-activation and purification, cow urine was made free of microbes thus, giving it massive toxic potential against drug resistant bacterial strains. Cow urine consists of few important components such as oestrogen (Biddle et al., 2007), phosphorous (Bravo et al., 2003), nitrogen (Yan et al., 2007), chloride (Coppock et al., 1979), potassium (Lebeda and Bus, 1997), calcium (Van-Leeuwen et al., 1976), urinary proteins (Gabel and Poppe, 1986) and pheromones (Tauck and Berardinelli, 2007). In India, cows are very important animal resource and are highly useful in agriculture and in the dairy industry (Jonker and Kohn, 2001). It has been observed that important forest dwelling cows secrete so many herbal compounds in urine, which are of high medicinal value. In such cows, plant dietary organic and inorganic compounds effectively get absorbed in the rumen and digested by bacterial activity. But there are some compounds, which are not disturb by any microbial enzyme action and are secreted in their natural form in

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cow urine. In the present investigation, the antimicrobial activity of photo activated cow urine against seven pathogenic bacterial strains that is, *Staphylococcus aureus, Bacillus cereus, Streptococcus pneumoniae, Escherichia coli, Klebsiella pneumoniae, Lactobacillus acidophilus* and *Micrococcus luteus* was investigated.

MATERIALS AND METHODS

Collection of cow urine

Fresh cow urine was collected from the local cow yard established within the vicinity of the University. For collection, adult female dwellers of more than 4 years of age were selected. For experimental use, fresh cow urine was photo activated for 144 h in sunlight by keeping it in sealed glass bottles. It was purified on a silica gel G-25 column and passed through two separate columns simultaneously to get rid of all the precipitated material and debris. Purified cow urine was stored at 4°C for long-term use. Before evaluation of antimicrobial activity, cow urine was tested for the presence of other pathogens microscopically as well as by the use of broth culture.

Bacterial culture

Cultures of seven pathogenic bacterial strains each of *E. coli* (ATCC 25922), *B. cereus* (ATCC 11778), *L. acidophilus* (ATCC 53103), *M. luteus* (ATCC 9341), *S. aureus* (ATCC 25923), *K. pneumoniae,* (ATCC 15380) and *S. pneumoniae* (ATCC 12755) were maintained in the laboratory in Luria broth (2%, w/v) regularly for four days at 37°C before used in experiments. For this purpose a portion (100 µl) of overnight bacterial culture was mixed in the tests and control for inoculation. For activity testing, bacterial cultures were stored at 4°C and sub cultured after every 3rd day in solid agar plates.

Determination of minimum inhibitory concentration (MIC) and minimum bacterial concentration (MBC) values

For determination of antimicrobial activity, bacterial growth inhibition was accessed in the presence of different increasing concentrations of cow urine. For this purpose, cow urine was diluted by using serial micro dilution method with Luria broth culture medium to a final concentration range of 32 - 0.0625 µl/ml. The tested essential oils were added to fresh suspension after following the serial dilutions up to 10^{-10}. Cow urine was assayed in triplicate. In another set of experiment, two important essential oils such as Neem and Bowchi oils were mixed in 1:1 ratio, separately. Before conducting the experiments, all conditions for *in vitro* anti-microbial activity were standardized to determine MIC and MBC values. The MIC values were considered as the lowest concentration of essential oil, which showed no turbidity in the culture flask after 24 h of incubation at 37°C. The turbidity in the culture flasks was considered as visible growth of microorganisms. Furthermore, it was standardized in terms of absorbance at 600 nm in a visible spectrophotometer. For the determination of MBC growth, inhibitory assays were performed. For this purpose, inoculum size was adjusted to prepare a final colony number of 10^8 colony forming units (CFU/ml) in a sterile agar plates. The incubation of test and control cultures was performed at 37°C for 24 h. For comparison, both negative and positive controls were set and the least concentration at which no visible growth was obtained in agar plates was considered as the MBC value.

Screening for antibacterial activity

Antimicrobial activity of cow urine was evaluated by agar disc diffusion method. For this purpose, solid agar- agar media was used. For antimicrobial activity testing a known volume (32 µl) of purified cow urine was coated on sterile filter paper discs (Whatmann No. 1) measuring 6 mm in size. These cow urine impregnated discs were made dry under laminar flow cabinet. Before the experiment, inoculum's size was determined and adjusted to prepare a final colony number of 10^8 CFU/ml in sterile agar plates. Bacterial inoculum was spread evenly on to the surface of the agar plate by sterile rubber pad spreader before cow urine coated discs were positioned on the inoculated agar surface. Besides, comparison combinations of cow urine with different essential oils were also used. Each experiment was assayed in triplicate. Sterile distilled water was used as negative control. Different antibiotics that is, tetracycline, ampicillin and ciprofloxcin were also coated on filter discs, separately. All treated and untreated plates were incubated for 24 h at 37°C. The antibacterial activity was assessed in agar plates based on the size of the inhibition zone diameter surrounding the filter paper discs.

RESULTS

The antimicrobial activity of photoactivated cow urine was tested against seven pathogenic strains of bacteria. The MIC value of photoactivated cow urine and essential oils are shown in Table 1a. The MIC value of photoactivated cow urine obtained was very low. The MIC value of binary combination of photoactivated cow urine with essential oil obtained was 0.25 µl /ml against *S. aureus* (ATCC 25923), *B. cereus* (ATCC 11778), *L. acidophilus* (ATCC 53103, *M. luteus* (ATCC 9341), while it was 0.125 µl /ml against *E. coli* and 0.5 µl/ml against *S. pneumoniae* (ATCC 12755) (Table 1b). The MIC value of Neem oil and cow urine was found to be 0.125 µl against *E. coli* which happened to be the lowest. The highest was 0.5 µl against *S. pneumoniae*. Neem oil alone showed the lowest MIC value of 0.25 µl against *B. cereus*, while it showed the highest MIC value of 2.0 µl against *S. aureus* and *E. coli* (2 µl). Similarly, Bawchi oil and cow urine mixture showed lowest MIC value of 0.125 µl against *S. aureus*, *B. cereus* and *L. acidophilus*, *M. luteus*, *S. pneumoniae* and *E. coli*. The only exception is *K. pneumoniae* in which MIC value was found to be 0.25 µl. Bawchi oil showed higher MIC value than Bawchi oil mixed with cow urine that is, 2.0 µl against *E. coli* and 1.0 µl against *B. cereus.* In this experiment, it was observed that photo-activated cow urine mixed with essential oil has synergistic activity as the antimicrobial activity was found to be significantly increased in each bacterial strain. Furthermore, photoactivated cow urine showed better bactericidal activity than the synthetic antibiotics. The MBC value in cow urine with neem oil was found be 0.5 - 1.0 µl. Similarly, Bawchi oil mixed with cow urine showed low MIC value against all bacterial strains except *S. pneumoniae* and *E. coli* (Table 2a). Growth inhibition was also observed in agar disc assays. It worked well in the presence of photoactivated cow urine and essential oils

Table 1a. MIC* values obtained in presence of cow urine and essential oils against seven pathogenic bacterial strain.

S/N	Bacterial strains	Oils and its combinations					Antibiotics		
		Neem oil	Bawchi oil	Photoactivated Cow urine	Neem oil + Cow urine	Bawchi oil + Cow urine	Tetracycline	Ampicillin	Ciprofloxin
1	*Staphylococcus aureus*	2.0	0.25	0.25	0.25	0.125	0.458	0.458	0.458
2	*Bacillus cereus*	0.25	1.0	0.125	0.25	0.125	0.915	0.458	0.458
3	*Lactophilus acidophilus*	0.5	0.25	1.0	0.25	0.125	0.458	0.229	0.229
4	*Micrococcus luteus*	4.0	0.5	0.25	0.25	0.125	0.458	0.915	0.229
5	*Klebsiella pneumoniae*	2.0	0.5	0.25	0.25	0.25	0.458	0.229	0.915
6	*Streptococcus pneumoniae*	0.5	0.5	0.125	0.5	0.125	0.458	0.114	0.458
7	*Escherichia coli*	2.0	2.0	0.125	0.125	0.125	0.458	0.458	0.195

*MIC = Minimum inhibitory concentration (µl/ml).

mixed. On the basis of size obtained in inhibition zone, bacterial growth was divided into three categories of resistant (> 7 mm), intermediate (> 12 mm) and susceptible (> 18 mm), respectively. Photo-activated cow urine showed high susceptibility against almost all seven bacterial strains used in the study, as the inhibition zone diameters obtained were more than 18 mm in size (Table 3). Inhibition zone diameter was obtained in the presence of photoactivated cow urine that is, 33-36 mm at 32 µl concentration. More specifically, highest growth inhibition zone of 41 mm was obtained in photoactivated cow urine and Bawchi oil against *K. pneumoniae*, at 32 µl concentration in agar disc diffusion method after hours (Table 3). However, cow urine showed synergistic effect when mixed with essential oils.

DISCUSSION

From the results, it is clear that photo activated cow urine showed very high susceptible to all bacterial strains at very low concentration. In the bioassays, photo activated cow urine exhibited a higher degree of antimicrobial activity in comparison to essential oils and antibiotics. When equilibrated amount of photo activated cow urine was mixed with Neem oil and Bowchi oil separately, it showed remark-able synergistic activity in bacterial cultures. Least MIC value was obtained in binary combinations of cow urine with essential oils, while photo activated cow urine showed 0.25 µl/ml. When these MIC values were compared with certain broad-spectrum antibiotics such as tetracycline, ampicilllin and ciprofloxacin, there was a higher MIC values than the natural products used.

Similarly, cow urine and its combinations showed least MBC values which showed higher susceptibility and synergistic effect of cow urine to bacterial cultures (Table 2b). Besides, effectiveness of binary combinations of essential oil and cow urine were also used to determine the growth inhibition zone diameter in agar disc diffusion assays. Maximum growth inhibition zone diameter was obtained in Neem oil (45 mm) against *S. pneumoniae*. Neem oil and cow urine showed 33 - 35 mm inhibition zone diameter against *B. cereus*, *L. acidophilus*, *M. luteus, K. pnemoniae* and *S. pneumoniae,* while Bawchi oil and cow urine combination showed high diameter inhibition zone diameter of 41 mm. Simple Bawchi oil alone showed inhibition zone diameters in a range of 16 -25 mm against all bacterial strains at 32 µl concentration, while photoactivated cow urine showed 32-36mm inhibition zone diameter homogeneously against all bacterial strains (Table 3).

This antimicrobial of cow urine may be due to presence of certain volatile organic ingredients found in cow urine which may get more toxicity after photo activations. Besides, after photo-activation, few biogenic volatile in-organic and organic compounds such as CO_2, NH_3, CH_4, methanol, propanol and acetone were also formed. Purification of cow urine on silica gel G- 25 column also significantly minimized the presence

Table 1b. Determination of MIC value cow urine and essential oils against pathogenic bacterial strain.

Oil	Conc. µl/ml	1	2	3	4	5	6	7	Positive control	Negative control
	32.0	-	-	-	-	-	-	-	+	-
	16.0	-	-	-	-	-	-	-	+	-
	8.0	-	-	-	-	-	-	-	+	-
	4.0	-	-	-	-	-	-	-	+	-
	2.0	-	-	-	-	-	-	-	+	-
	1.0	-	-	-	-	-	-	-	+	-
	0.5	-	-	-	-	-	-	-	+	-
	0.25	-	-	-	-	-	+	-	+	-
	0.125	+	+	+	+	+	+	-	+	-
	0.0625	+	+	+	+	+	+	+	+	-
	32	-	-	-	-	-	-	-	+	-
	16	-	-	-	-	-	-	-	+	-
	8	-	-	-	-	-	-	-	+	-
	4	-	-	-	-	-	-	-	+	-
	2	-	-	-	+	-	-	-	+	-
	1	+	-	-	+	+	-	+	+	-
	0.5	+	-	-	+	+	-	+	+	-
	0.25	+	-	+	+	+	+	+	+	-
	0.125	+	+	+	+	+	+	+	+	-
	0.0625	+	+	+	+	+	+	+	+	-
	32.0	-	-	-	-	-	-	-	+	-
	16.0	-	-	-	-	-	-	-	+	-
	8.0	-	-	-	-	-	-	-	+	-
	4.0	-	-	-	-	-	-	-	+	-
cow urine	2.0	-	-	-	-	-	-	-	+	-
	1.0	-	-	-	-	-	-	-	+	-
	0.5	-	-	-	-	-	-	-	+	-
	0.25	-	-	-	-	-	-	-	+	-
	0.125	-	-	-	-	+	-	-	+	-
	0.0625	+	+	+	+	+	+	+	+	-

Table 1b. Contd.

Oil	Conc. µl/ml	1	2	3	4	5	6	7	Positive control	Negative control
	32.0	-	-	-	-	-	-	-	+	-
	16.0	-	-	-	-	-	-	-	+	-
	8.0	-	-	-	-	-	-	-	+	-
	4.0	-	-	-	-	-	-	-	+	-
	2.0	-	-	-	-	-	-	-	+	-
	1.0	-	-	-	-	-	-	+	+	-
	0.5	-	+	-	-	-	-	+	+	-
	0.25	-	+	-	+	+	+	+	+	-
	0.125	+	+	+	+	+	+	+	+	-
	0.0625	+	+	+	+	+	+	+	+	-
	32.0	-	-	-	-	-	-	-	+	-
	16.0	-	-	-	-	-	-	-	+	-
	8.0	-	-	-	-	-	-	-	+	-
	4.0	-	-	-	-	-	-	-	+	-
	2.0	-	-	-	-	-	-	-	+	-
	1.0	-	-	-	-	-	-	-	+	-
	0.5	-	-	+	-	-	-	-	+	-
	0.25	-	-	+	-	-	-	-	+	-
	0.125	+	-	+	+	+	-	-	+	-
	0.0625	+	+	+	+	+	+	+	+	-
	57.14	- - -	- - -	- - -	- - -	- - -	- - -	- - -	+	-
	28.57	- - -	- - -	- - -	- - -	- - -	- - -	- - -	+	-
	14.28	- - -	- - -	- - -	- - -	- - -	- - -	- - -	+	-
	7.14	- - -	- - -	- - -	- - -	- - -	- - -	- - -	+	-
	3.57	- - -	- - -	- - -	- - -	- - -	- - -	- - -	+	-
	1.78	- - -	- - -	- - -	- - -	- - -	- - -	- - -	+	-
	0.892	- - -	- - -	- - -	- - -	- - -	- - -	- - -	+	-
	0.446	+ - -	+ - +	+ - -	- + -	+ - +	- - +	+ - -	+	-
	0.223	+ + +	+ + +	+ - -	+ + -	+ - +	+ - +	+ + +	+	-
	0.1116	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+	-

- = Absence of growth; + = presence of growth, positive control: bacterial suspension and media; negative control: toxin and broth; 1 = *Staphylococcus aureus;* 2 = *Bacillus cereus;* 3 = *Lactobacillus acidophilous;* 4 = *Micrococcus luteus;* 5 = *Klebsiella pneumonia;* 6 = *Streptococcus pneumonia;* 7 = *Escherichia coli.*

Table 2a. MBC* values obtained in presence of photo-activated cow urine and essential oils against pathogenic bacterial strains.

S/N	Bacterial strains	Oils and its combinations					Antibiotics		
		Neem oil	Bawchi oil	Photoactivated Cow urine	Neem oil + Cow urine	Bawchi oil + Cow urine	Tetracy cline	Ampicillin	Ciprofloxin
1	*Staphylococcus aureus*	1.0	1.0	0.5	0.5	1.0	0.915	0.915	0.915
2	*Bacillus cereus*	1.0	2.0	0.25	0.5	0.25	0.915	1.83	1.83
3	*Lactophilus acidophilus*	2.0	2.0	0.5	0.5	1.0	0.915	0.915	3.66
4	*Micrococcus luteus*	0.25	2.0	0.25	0.5	0.25	7.32	7.32	0.915
5	*Klebsiella pneumoniae*	2.0	2.0	0.5	0.5	1.0	0.915	1.83	1.83
6	*Streptococcus pneumoniae*	1.0	1.0	1.0	1.0	0.25	0.114	0.915	0.915
7	*Escherichia coli*	0.25	0.25	1.0	0.25	0.25	0.915	1.83	1.83

* Minimum bactericidal concentration

Table 2b. Determination of MBC values of photoactivated cow urine and essential oils against pathogenic bacterial strains.

i) Bacterial strains.

Oils	1	2	3	4	5	6	7
Neem oil + cow urine	0.5	0.5	0.5	0.5	0.5	1	0.25
Neem oil	1	1	2	0.25	2	1	0.25
Bawchi oil + Cow urine	1	0.25	1	0.25	1	0.25	0.25
Bawchi oil	1	2	2	2	2	1	0.25
Photoactivated cow urine	0.5	0.25	0.5	0.25	0.5	1	1

1 = *Staphylococcus aureus;* 2 = *Bacillus cereus;* 3 = *Lactobacillus acidophilous;* 4 = *Micrococcus luteus;* 5 = *Klebsiella pneumonia;* 6 = *Streptococcus pneumonia;* 7 = *Escherichia coli.*

ii) Antibiotics

Bacterial strains	Antibiotics	Concentrations (mg/ml)									
		57.14	28.57	14.28	7.14	3.57	1.78	0.892	0.446	0.233	0.1116
		1 2 3	1 2 3	1 2 3	1 2 3	1 2 3	1 2 3	1 2 3	1 2 3	1 2 3	1 2 3
K. pneumoniae	Aqueous	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - +	+ - +	+ + +
E. coli	Aqueous	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - +	+ + +	+ + +
S. aureus	Aqueous	- - -	- - -	- - -	- - -	- - -	- - -	- - -	+ - -	+ + +	+ + +
L. acidophilus	Aqueous	- - -	- - -	- - -	- - -	- - -	- - -	- - -	+ - +	+ - -	+ + +
M. luteus	Aqueous	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	+ - -	+ + +
S. pneumoniae	Aqueous	- - -	- - -	- - -	- - -	- - -	- - -	- - -	- - -	+ - +	+ + +
B. cereus	Aqueous	- - -	- - -	- - -	- - -	- - -	- - -	- - -	+ - -	+ + +	+ + +

- = Absence of growth; + = presence of growth, positive control: bacterial suspension and media; negative control: toxin and broth; 1 = tetracycline; 2 = ampicillin; 3 = ciprofloxin.

Table 3. Anti-microbial activity of different essential oils measured agar diffusion method. The effectiveness of different essential oils is demonstrated by the size of the micro- organism growth inhibition zone around the filter paper disc, which is typically expressed as the diameter of the zone in mm.

Essential oil + Cow urine	Concentration used per disc	Disc diffusion diameter (in mm)						
		Staphylococcus aureus	***Bacillus cereus***	***Lactobacillus acidophilus***	***Micrococcus luteus***	***Klebsiella pneumoniae***	***Streptococcus pneumoniae***	***Escherichia coli***
Control		-----	----	-----	----	----	----	----
Neem oil + Cow urine	32 µl	27	34	35	3	33	35	26
Neem oil	32 µl	24	19	23	20	23	45	16
Bawchi oil + cow urine	32 µl	31	28	27	33	41	36	31
Bawchi oil	32 µl	19	16	23	23	16	21	23
Photoactivated cow urine	32 µl	34	36	33	35	36	33	35
Tetracycline	40 µg	23	20	24	25	8.0	24	24
Ampicillin	40 µg	23	15	16	18	8.0	20	20
Ciprofloxin	40 µg	24	24	26	23	8.0	24	22

Control = No antibiotic.
*The strength of activity is presented as resistant (> 7 mm), intermediate (> 12 mm) and susceptible (> 18 mm).

of microbes. Furthermore, re-filtration and centrifugation at high speed 15,000 X g also made cow urine almost free of any infection. In the experiments, it was observed that photoactivated cow urine mixed with essential oil worked effectively as bactericidal agent. This potential was also observed after 12 months of stored cow urine in sterilized plastic containers. Furthermore, after 144 h continuous photo-activation, no bacterial activity was observed. It may be due to presence of more number of cations and formation of nitrosamines (Stephany and Schuller, 1978). Moreso, some metabolic secondary nitrogenous products may be formed during photo activation, which may increase the bactericidal activity of cow urine. In addition, photoactivation of cow urine became highly acidic in comparison to fresh cow urine and showed significant decrease in pH and cationanion difference (Hu et al., 2007). Moreso, inorganic phosphorus, chloride and dimethylamine may also play an important role in bacteria lkilling (Kurosaki et al., 2007). However, acidic cow urine proved highly bactericidal. Contrary to this, fresh cow urine did not function as a bactericidal agent but photoactivated cow urine has shown enormous lethality in bacterial cultures. It may also be possible that hydrolytic state of cow urine and presence of amino acids and urinary peptides may enhance bacterial killing (Badadani et al., 2007) by increasing the bacterial cell surface hydrophobicity. Furthermore, antibacterial activity of cow may be increased due to formation of some reactive compounds such as formaldehyde, sulfinol, ketones and some amines during photo activation and long-term storage (Turi et al., 1997). It can be conclude that photoactivated cow urine and essential oil in single and binary combination became more toxic and can work more effectively than the antibiotics drugs and are proved as good source of antimicrobial agents.

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