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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 and it binary combinations was determined against seven bacterial strains. Photoactivated cow urine has shown MIC value 0.25 µl/ml MIC value against *Staphylococcus aureus* (ATCC 25923), *Bacillus cereus* (ATCC 11778), *Lactobacillus acidophilus* (ATCC 53103 and *Micrococcus luteus* (ATCC 9341), while it was found 0.125 µl/ml against *E. coli* (ATCC 25922). Binnary combinations of cow urine with Neem and Bavchi oil has shown synergistic effect as the MIC value obtained was 0.125-0.25 µl/ml. Similarly photoactivated cow urine has shown least MBC value that is, 0.25-1.0 µl/ml. Further, growth inhibition zone diameters obtained in presence of photoactivated cow urine and its binary combinations were found much larger than antibiotic drugs. Neem oil has shown highest inhibition zone diameter that is, 45 mm against *S. pneumoniae*. Neem oil and cow urine have shown 33 to 35 mm inhibition zone diameter against *B. cereus, L. acidophilus, Micrococcus. Luteus, K. pneumoniae* and *S. pneumoniae,* while Bawchi oil and cow urine combination has shown high diameter inhibition zone diameter that is 41 mm against *K. pnumoniae.* Photoactivated cow urine has shown 32 to 36 mm inhibition zone diameter homogeneously against all bacterial strains. It proves very high antimicrobial susceptibility of cow urine and essential oils.

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

INTRODUCTION

For 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 enhance drug threshold level in microbes. Therefore, to combat the problem of microbial infection and drug resistance new alternative of synthetic drugs have been explored, though antimicrobial activities of so many natural products have been explored. But there is no report available on antimicrobial activity of cow urine. In spite of the fact that the cow urine has great pharmacological importance and has great aesthetic and medicinal value though it's utility has been mentioned in holy texts of Indian literature. Cow urine has certain volatile and non-volatile components, which might have very high anti-microbial activity (Shaw et al., 2007). After photo-activation and purification cow urine was made free from microbes and it gains massive toxic potential to kill drug resistant bacterial strains. Cow urine consists few important components such as estrogen (Biddle et al., 2007), Phosphorous (Bravo et al., 2003), Nitrogen (Yan et al., 2007), chloride (Coppock et al., 1979), Potassium (Lebeda and Bus, 1997) and Calcium (Van-Leeuwen et al., 1976) urinary proteins (Gabel et al., 1986) and pheromones (Tauck and Berardinelli, 2007). In India, cows are very important animal resource and are highly useful in agriculture and 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 origin dietary organic and inorganic compounds effectively get absorbed in the rumen and digested by bacterial activity. But there are some compounds, which do not disturb by any

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any microbial enzyme action and secreted in their natural form in cow urine. In the present investigation we have observed the antimicrobial activity of photo activated cow urine against seven pathogenic bacterial strains that is, *Staphylococcus aureus, Bacillus cereus, Streptococcus pneumoniae, Escherichia coli, Klebisella pneumoniae, Lactobacillus acidophilus* and *Micrococcus luteus.*

MATERIALS AND METHODS

Collection of Cow urine

Fresh cow urine was collected from local Cow yard established in the vicinity of University area. For collection adult female dwellers having age of more than 4 years were selected. For experiments 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 presence of other pathogens microscopically as well as in broth culture.

Bacterial culture

Cultures of seven pathogenic bacterial strains each of *Escherichia coli* (ATCC 25922), *Bacillus cereus* (ATCC 11778), *Lactobacillus acidophilus* (ATCC 53103), *Micrococcus luteus* (ATCC 9341), *Staphylococcus aureus* (ATCC 25923), *Klebsiella pneumoniae,* (ATCC 15380) and *Streptococcus 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 bactericidal 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 at a final concentration range from 32 to 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 experiments all the conditions for *in vitro* anti-microbial activity were standardized to determine MIC and MBC values. The MIC values considered as the lowest concentration of essential oil, which have shown 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. Further, it was standardized in terms of absorbance at 600 nm in a visible spectrophotometer. For the determination of MBC value growth inhibitory assays were performed. For this purpose inoculum size was adjusted to prepare a final colony number as 10^8 colony forming units (CFU/ml) in 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 MBC value.

Screening of antibacterial activity

Antimicrobial activity of cow urine was also evaluated by agar disc diffusion method. For this purpose solid agar-agar media was used. For antimicrobial activity testing a known volume of purified cow urine that is, 32 µl 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 experiment inoculum's size was determined and adjusted to prepare a final colony number as 10^8 colony forming units (CFU/ml) in sterile agar plates. Bacterial inoculum was spread evenly on to the surface of agar plate by sterile rubber pad spreader before cow urine coated discs were positioned on the inoculated agar surface. For comparison various 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 1. The MIC value of photoactivated cow urine obtained is very low. The MIC value of binary combination of photoactivated cow urine with essential oil was obtained 0.25 µl /ml against *Staphylococcus aureus* (ATCC 25923), *Lactobacilus acidophilus* (ATCC 53103, *Micrococcus luteus* (ATCC 9341), while it was found 0.125 µl /ml against *E. coli* and 0.5 µl/ml against *Streptococcus pneumoniae* (ATCC 12755) and *Bacillus cereus* (ATCC 11778) (Table 1) .The MIC value of Neem oil and cow urine was found lowest 0.125 µl against *Escherichia coli*, while highest 0.5 µl against *Streptococcus pneumoniae*. Neem oil alone has shown lowest MIC value 0.25 µl against *Bacillus cereus*, while it has shown highest MIC value that is, 1.0 µl against *K. pneumoniae* and *E. coli* (1.0 µl). Similarly Bawchi oil and cow urine mixture has shown lowest MIC value 0.125 µl against *Staphylococcus aureus*, *Bacillus cereus* and *Lactobacillus acidophilus*, *Micrococcus luteus*, *Streptococcus pneumoniae* and *Escherichia coli*. Only exception is *Klebsiella pneumoniae* in which MIC value was found to be 0.25 µl. Bawchi oil has showed higher MIC value that is, 1.0 µl against *B. cereus* and *E. coli* than Bawchi oil mixed with cow urine that is, 1.0 µl against *Escherichia coli* and *Bacillus cereus.* In this experiment it was observed that photoactivated cow urine mixed with essential oil showed synergistic activity as the antimicrobial activity was found to be significantly increased in each bacterial strain. Further, photoactivated cow urine has shown better bactericidal activity than the synthetic antibiotics. The MBC value in cow urine with neem oil was found to be 0.25 to 1.0 µl. Similarly Bawchi oil mixed with cow urine has shown low MIC value against all bacterial strains except *Streptococcus*

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

S. No.	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*	1.0	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*	2.0	0.5	0.25	0.25	0.125	0.458	0.915	0.229
5	*Klebsiella pneumoniae*	1.0	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*	1.0	1.0	0.125	0.125	0.125	0.458	0.458	0.195

* MIC = minimum inhibitory concentration (µl/ml)

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

S. No.	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	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	1.0	0.5	1.0	0.915	0.915	3.66
4	*Micrococcus luteus*	4.0	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	0.892	0.915	0.915
7	*Escherichia coli*	2.0	2.0	1.0	0.25	0.25	0.915	1.83	1.83

* Minimum bactericidal concentration

pneumoniae and *Escherichia coli* (Table 2). Besides this, growth inhibition was also observed in agar disc diffusion assays. It has worked well in presence of photoactivated cow urine and essential oils mixed. On the basis of size obtained in inhibition zone bacterial growth was divided in to three categories, that is resistant (>7 mm), intermediate (> 12 mm) and susceptible (>18 mm) respectively. Photo-activated cow urine has shown 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 presence of photo-activated cow urine that is 33 to 36 mm at 32 µl concentration. More specifically, highest growth inhibition zone was obtained in photoactivated cow urine and Bawchi oil that is 41 mm against *Klebsiella pneumoniae,* at 32 µl concentration in

Table 3. Anti-microbial activity of different essential oils measured by agar disc 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***
Neem oil + Cow urine	Control	-----	----	-----	----	----	----	----
	32 µl	27	34	35	30	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).

agar disc diffusion assay after 24 h (Table 3).

DISCUSSION

From the results it is clear that photo activated cow urine has shown very high susceptibility to all bacterial strains at a very low concentration. In the bioassays photo activated cow urine exhibited a higher degree of anti-microbial activity in comparison to essential oils and broad-spectrum antibiotics. When equilibrated amount of photo activated cow urine was mixed with neem oil and Bawchi oil separately, it showed remarkable synergistic activity in bacterial cultures. Least MIC value that is, 0.125 µl/ml was obtained in binary combinations of cow urine with Bawchi oil, while photo activated cow urine has shown 0.25 µl/ml. When these MIC values were compared with certain broad-spectrum antibiotics such as tetracycline, ampicilllin and ciprofloxacin has shown higher MIC values in antibiotics than the natural products used (Table 1).

Similarly cow urine and its combinations have shown least MBC values which again proves higher susceptibility and synergistic effect of cow urine to bacterial cultures (Table 2). Besides this, 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 *Streptococcus pneumoniae*. Neem oil and cow urine has shown 33 to 35 mm inhibition zone diameter against *B. cereus*, *Lactobacilus acidophilus*, *Micrococcus luteus, Klebsiella pnemoniae*, and *Streptococcus pneumoniae,* while Bawchi oil and cow urine combination has shown larger inhibition zone diameter that is 41 mm against *K. pnemoniae*. Simple Bawchi oil alone has shown inhibition zone diameters in a range of 15 - 25 mm against all bacterial strains at 32 µl concentration, while photo-activated cow urine has shown 32 to 36 mm inhibition zone diameter homogeneously against all bacterial strains (Table 3).

This antimicrobial activity of cow urine may be due to presence of certain volatile organic ingredients found in cow urine which may gain more toxic after photo activation. Besides this, after photo-activation few biogenic volatile inorganic and organic compounds such as CO_2, NH_3, CH_4, methanol, propanol and acetone are also formed. Purification of cow urine on silica gel G-25 column has also significantly minimized the presence of microbes. Further, re-filtration and centrifugation at high speed 15,000 X g for 45 min at 3°C also made cow urine almost free from any infection. In the experiments, it was observed that photoactivated cow urine mixed with essential oil work as more effective bactericidal agent. This potential was also observed after 12 months of stored cow urine in sterilized plastic containers. Further, 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). Besides this, some metabolic secondary nitrogenous products may be formed during photo activation, which may increase the bactericidal activity of cow urine. Further, photoactivation cow urine become highly acidic in comparison to fresh cow urine and shows significant decrease in pH and cation-anion difference (Hu et al., 2007). Besides this, inorganic phosphorus, chloride and dimethylamine may also play an important role in bacterial killing (Kurosaki et al., 2007). However, acidic cow urine is proved highly bactericidal. Contrary to this, fresh cow urine did not

function as a bactericidal agent but photo-activated 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 the bacterial killing (Badadani et al., 2007) by increasing the bacterial cell surface hydrophobicity. Further, antibacterial activity of cow may be increased due to formation of some reactive compounds such as formaldehyde, sulfinol, kitones and some amines during photo activation and long-term storage (Turi et al., 1997). It can be concluded that photo-activated cow urine and essential oil in single and binary combination become more toxic and can work more effectively than the antibiotics drugs and are proved as good source of antimicrobial agents.

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