

ANTIMICROBIAL PROPERTY OF A HERBAL PREPARATION CONTAINING *DALBERGIA SISSOO* AND *DATURA STRAMONIUM* WITH COW URINE AGAINST PATHOGENIC BACTERIA

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In this study, a herbal preparation containing *Dalbergia sissoo* and *Datura stramonium* with cow urine (DSDS), was evaluated for its antibacterial potential against pathogenic strains of gram-positive (*Staphylococcus aureus* and *Streptococcus pneumoniae*) and gram-negative (*Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*) bacteria. Antibacterial activity was compared to standard antibiotic drugs i.e. Chloramphenicol (30 mcg), Ampicillin (10 mcg), Nalidixic acid (10 mcg) and Rifampicin (30 mcg). Cow urine extract was found to be most active against both gram-positive as well as gram-negative bacteria. Clinical isolate of *S. aureus* showed higher sensitivity towards cow urine extract of DSDS than standard strains, and inhibited growth on most regulatory levels such as inhibition of protein, DNA, RNA and peptidoglycan synthesis. The results of the present study shows that the cow urine extract of DSDS may be used as a potent antiseptic preparation for prevention and treatment of chronic bacterial infections.

Bacterial infections are an emerging problem worldwide, especially in developing countries, such as India (1). Gram-positive bacteria, such as *Staphylococcus aureus*, are mainly responsible for postoperative wound infection, toxic shock syndrome, scaled skin syndrome, septicemia, endocarditis, osteomyelitis, and food poisoning (2). *Streptococcus pneumoniae* causes lobar pneumonia, bronchopneumonia, bronchitis, endocarditis, sinusitis, and conjunctivitis (3). Gram-negative

bacteria such as *E. coli*, which resides in human intestine, causes lower urinary tract infection, cholecystitis or septicemia, and another strain, *Klebsiella pneumoniae*, causes chest infections, urinary infections and wound infections (4-5). Various antibiotics are available on the market to treat the bacterial infectious diseases by working on various targets to inhibit pathogen growth. Gram-positive and gram-negative bacteria can be inhibited by antibiotics viz. Chloramphenicol, Nalidixic acid,

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Rifampicin and Ampicillin, either by blocking protein, DNA, RNAs or peptidoglycan synthesis, respectively (6). However, the development of bacterial resistance to presently available antibiotics enforces the search for new antibacterial agents (7-8). WHO emphasized research for natural components from herbal medicines to find new antibacterial agents (9).

Medicinal plants have been the part and parcel of human society to combat diseases since the dawn of civilization. The earliest description of curative properties of medicinal plants were described in the Rigveda (2500-1800 BC), Charak Samhita and Sushruta Samhita. Herbal medicine remains one of the most common forms of therapy widely available throughout the world population (10-12). *Dalbergia sissoo* (English: South Indian rosewood; Hindi: Sisam) is distributed in Baluchistan, Wazaristan, and W. Himalayas up to 4,000 ft, Terai of Nepal and Sikkim to Upper Assam; extensively planted throughout India. Its bark and wood are bitter, hot, acrid, aphrodisiac; its properties are: abortifacient, expectorant, anti-helminthic, anti-pyretic, appetizer; allays thirst, vomiting, burning sensation; cures skin diseases, troubles of the anus, ulcers, diseases of the blood, leukoderma, dyspepsia, dysentery. The juice of its leaves is good for eye diseases (13). *D. sissoo* has a wide spectrum of medicinal use according to the Indian Ayurveda system. In recent years, various biochemical and medicinal properties of *D. sissoo* have also been reported (14-19), but none of these reported anti-microbial potentials of preparations from this plant. *Datura stramonium* (English: Devil's apple, Hindi: Dhatura) is distributed from Kashmir to Sikkim up to 8,000 ft, Baluchistan and hilly districts of central and South India, and throughout the temperate and warmer regions of the world. Its seeds have an acrid, bitter and sharp taste, and have been used overheating, tonic febrifuge, antihelminthic, alexiteric, emetic, biliousness, jaundice, piles (13). Moreover, cow urine alone is already used in Indian traditional medicinal systems and also combined with many other plants to treat various diseases i.e. infections, diabetes, cancer and immune diseases (20-22).

The present study is a research on the antibacterial effects of DSDDS (a herbal preparation containing *D. sissoo*, *Datura stramonium* and gau-mutra) against

pathogenic bacteria such as *Staphylococcus aureus*, *Streptococcus pneumoniae*, *E. coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and other clinical isolated strains. This effect was compared with various antibiotics i.e. Ampicillin, Nalidixic acid, Rifampicin and Chloramphenicol with different mechanisms and the action of pure cow urine on bacteria.

MATERIALS AND METHODS

Collection and maintenance of micro-organisms

S. aureus standard (SR; ATCC 25923) and clinical isolate, *Streptococcus pneumoniae* (clinical isolate), *E. coli* standard (SR; ATCC 25922) and clinical isolate, *P. aeruginosa* (SR; ATCC 27853) and clinical isolate, and *K. pneumoniae*, (lab strain) were used as test organisms, and were obtained from the Department of Applied Microbiology, Cancer Hospital and Research Institute, Gwalior, M.P., India. Bacterial strains were maintained in nutrient broth, and sub-cultured for 14 days. All the organisms were characterized and confirmed by light microscopy as well as biochemical tests (IMVIC). Nutrient broth and nutrient agar, Mc-Conkey agar, (Hi-Media Mumbai), and other chemicals were obtained from E-Merck (Mumbai, India), Hi-Media (Mumbai, India), BDH (Mumbai, India) and Qualigens (Mumbai, India) of analytical grade.

Collection of plant parts and cow urine

Fresh plants were obtained from the local rural area of Datia (Uchad Forests) and from the fields of the Gwalior, Madhya Pradesh, India (Central India). The plant parts were dried in a cool, dark place. After complete drying, the plant parts were crushed to powder form and 200 g (100-100 g from each) powder was dissolved with different solvents to prepare plant extracts as described below. Cow urine was collected from a local dairy farm. Animals (age 5-10 years) were maintained on the normal feed and water, and fed *ad-libitum*. Pregnant cows 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), after discarding the first flow, and carried to the laboratory in an ice box. In the laboratory, the cow urine was passed through a 0.22 μm sterile filter unit, and aliquots were frozen at -20°C until further use (within one month).

Preparation of extracts

Four types of extracts were prepared of DSDDS, i.e. fraction I with water, fraction II with cow urine, fraction III with methanol, and fraction IV with chloroform. Dried

bark of *D. sisso* (100 gm) and seeds of *D. stramonium* (100 gm) were finely ground in a mortar and pestle by adding a little amount of corresponding solvent, and mixed finally in 200 ml of solvent and left overnight on stirrer. Residues in liquid mixtures of DSDS and solvents were filtered. Residues were re-extracted with corresponding solvents and finally filtrates were lyophilized under vacuum to give corresponding extracts (Fig. 1).

Anti-microbial potential assessment

Antimicrobial tests were then carried out by the disc-diffusion method (23). The bacterial strains were grown on nutrient broth at 37°C for 12-14 h and were maintained on nutrient agar slants at 4°C. Extracts were dissolved in ethylene glycol. The membrane filters (0.47 µm) were sterilized. Antimicrobial potential of different extract fractions were assessed using a disc diffusion method. A concentration of 2000 µg/disc was chosen, based on available literature. Sterile 6 mm diameter filter paper discs were impregnated with 2000 µg of the sterile test material and placed onto nutrient agar surface spread with 0.1 ml of bacterial culture. Plates were incubated at 37°C for 12-14 h. Experiments were carried out in triplicate. The results (mean value n = 3) were recorded by measuring the zone of growth inhibition around the discs. Control discs contained only ethylene glycol. For comparison, standard antibiotics Chloramphenicol (30 mcg), inhibiting bacterial protein synthesis, Nalidixic Acid (10 mcg), inhibiting DNA synthesis, Rifampicin (30 mcg), inhibiting RNA synthesis and Ampicillin (10 mcg), inhibiting bacterial cell wall biosynthesis, were incubated in the assay.

Statistical analysis

The values are presented as means ± SEM of triplicate samples. Data were subjected to analysis of variance, and significant differences among means of different groups were analysed using Student's t-test. The differences with $p < 0.05$ were considered statistically different.

RESULTS

In the present study, pure cow urine and various extracts (Fig. 1), i.e. water extract (fraction I), cow urine extract (fraction II), methanol extract (fraction III) and chloroform extract (fraction IV) were tested for antibacterial activity *in-vitro*. The antibacterial spectra showing inhibition in millimeters and as percentage (calculated by taking Chloramphenicol as positive control with 100% inhibition) for gram-positive and gram-negative bacteria are shown in Tables I and II respectively. It can be clearly seen in the results shown in Table I that all the fractions were

nearly inactive against gram-positive *S. aureus* and *S. pneumoniae*, except fraction II (cow urine extract) and fraction I (water extract). In *S. aureus*, clinical isolate fraction II showed more susceptibility than standard antibiotics (Table I). Among gram-negative bacteria, fraction II also displayed moderate activity against *E. coli*, *S. typhimurium* and *K. pneumoniae* (lab strain), while all other extracts were inactive. Interestingly, the pure cow urine also exhibited a good anti-microbial potential and its effects were approximately similar to the water extract of the DSDS. These results indicate that the water extract of DSDS was able to significantly inhibit the growth of the gram-positive and gram-negative bacterial strains, and other fractions, such as methanol and chloroform, were mostly inactive and did not demonstrate any significant activity. This demonstrates that DSDS can potentially inhibit pathogenic bacterial growth.

Furthermore, we also studied the minimum inhibitory concentration (MIC) for cow urine extract only (Fraction II) and results compared with standard antibiotics. It was observed that dilution decreased the activity gradually in gram-positive bacteria to 85% and 60% against *S. aureus* (SR) at 1/10 and 1/100 dilutions respectively, however *S. aureus* (CI) did not show significant change (93% at 1/10 dilution, although activity decreased to 77% at 1/100 dilution). In *S. pneumoniae* activity is decreased with 1/10 and 1/100 dilutions to 94% and 66% respectively (Table III). The gram-negative *E. coli* (SR), *P. aeruginosa* (SR) and *K. pneumoniae* (lab strain) showed a decrease in activity with dilutions. No activity was observed below 200 µg/disc concentration (1/10 dilution) against *E. coli*. The activity of fraction II against *E. coli* (SR) and *P. aeruginosa* (CI) was not affected significantly up to 1/10 dilution, but slightly decreased at 1/100 dilution. Moreover, cow urine fraction was also more effective against gram-positive compared to gram-negative strains. *S. aureus* (CI) and *S. pneumoniae* showed 213% and 95% inhibition respectively, compared to gram-negative strains of *E. coli* (CI), *P. aeruginosa* (CI) and *K. pneumoiae*, which showed inhibition of 35%, 23% and 37%, respectively. However, no significant activity ($p > 0.1$) was shown in standard reference strains of both gram-positive and gram-negative bacteria. This indicates that fraction II has

Table I. Zone of inhibition for various extracts of DSDS compared to reference drugs: activity against gram-positive bacteria.

Name of Drug ↓	Microorganisms					
	<i>Staphylococcus aureus SR</i>		<i>Staphylococcus aureus CI</i>		<i>Streptococcus pneumoniae CI</i>	
	Zone of inhibition		Zone of inhibition		Zone of Inhibition	
	In mm Mean	As percentage	In mm mean	As percentage	In mm Mean	As Percentage
Chloramphenicol 30 mcg	20.56±1.36	100	12.35±0.56	100	15.56±0.21	100
Nalidixic acid 10 mcg	9.33±0.86	45	7.97±1.35	64	7.35±0.45	47
Rifampicin 30 mcg	12.33±0.81	59	9.33±.21	75	14.00±0.35	89
Ampicillin 10 mcg	14.00±0.33	68	10.91±0.52	88	6.13±0.45	39
Pure gau-mutra	9.97±0.0.52	47	13.87±0.21	122	13.90±0.86	84
Fraction I	9.59±0.56	46	13.19±0.85	106	7.35±0.56	47
Fraction II	25.38±0.21	204	26.33±0.45	213	24.85±0.41	195
Fraction III	12.97±0.33	63	7.35±0.33	59	7.23±0.52	12
Fraction IV	10.83±05	54	7.00±0.04	56	7.30±0.03	13
Ethylene Glycol	6.00±0.00	00	6.00±0.00	00	6.00±0.00	00

SR: Standard reference; CI, Clinical isolates

Mean, Mean value of diameter of inhibition zone with standard error

^φ Percentge was calculated after substracting disc diameter (6 mm) from all abservations

Table II. Zone of inhibition for various extracts of DSDS compared to reference drugs: activity against gram-negative bacteria.

Name of Drug ↓	Microorganisms									
	<i>Escherichia coli SR</i>		<i>Escherichia coli CI</i>		<i>Klebsiella pneumoniae</i>		<i>Pseudomonas aeruginosa SR</i>		<i>Pseudomonas aeruginosa CI</i>	
	Zone of inhibition		Zone of inhibition		Zone of Inhibition		Zone of Inhibition		Zone of Inhibition	
	In mm Mean	As Percentage	In mm Mean	As Percentage	In mm Mean	As Percentage	In mm Mean	As percentage	In mm Mean	As Percentage
Chloramphenicol 30 mcg	24.00±0.57	100	14.33±0.25	100	26.25±0.33	100	26.33±0.87	100	21.33±0.87	100
Nalidixic acid 10 mcg	16.00±0.51	55	6.00±0.00	00	7.42±0.16	7	6.33±0.32	1	6.00±0.00	00
Rifampicin 30 mcg	20.33 ±0.87	68	6.00±0.00	00	6.00±0.00	00	6.00±0.00	00	6.00±0.00	00
Apmicillin 10mcg	15.66±0.87	53	6.52±0.65	6	6.00± 0.00	00	6.00±0.00	00	6.00±0.00	00
Pure Gau-Mutra	8.25±0.21	12	7.89±0.56	22	9.33±0.16	16	8.55±0.35	12	8.52±0.41	16
Fraction I	8.16±0.85	12	8.10±0.35	25	7.54±0.33	7	7.00±0.57	4	7.66±0.00	10
Fraction II	8.56±0.36	14	8.98±0.15	35	13.56±0.89	37	9.33±0.32	16	9.66±0.33	23
Fraction III	6.00±0.00	00	6.00±0.00	00	6.00±0.00	00	6.00±0.00	00	6.12±0.00	0.7
Fraction IV	6.00±0.00	00	6.00±0.00	00	6.00±0.00	00	6.00±0.00	00	6.00±0.00	00
Ethylene Glycol	6.00±0.00	00	6.00±0.00	00	6.00±0.00	00	6.00 ±0.00	00	6.00±0.00	00

Table III. Minimum inhibitory concentration of fraction II (gau-mutra extract) on gram-positive bacteria with Chloramphenicol as standard reference.

Name of Drug	Microorganisms					
	<i>Staphylococcus aureus</i> SR		<i>Staphylococcus aureus</i> CI		<i>Streptococcus pneumoniae</i> CI	
	Zone of inhibition		Zone of inhibition		Zone of Inhibition	
	In mm Mean	As percentage	In mm mean	As percentage	In mm Mean	As Percentage
Chloramphenicol 30 mcg	19.33±0.45	100	11.23±0.12	100	18.33±0.23	100
Fraction II	10.63± 0.33	55	27.53±0.33	245	16.98±0.11	92
1/10 dilution of fraction II	9.08± 0.015	47	24.59±0.22	218	15.97±0.22	94
1/100 dilution of fraction II	6.37± 0.011	33	21.23±0.45	189	11.35±0.01	67
Ethylene glycol	6.00±0.00	00	6.00±0.00	00	6.00±0.00	00

Table IV. Minimum inhibitory concentration of fraction II (gau-mutra extract) on gram-negative bacteria with Chloramphenicol as standard reference.

Name of Drug	Microorganisms									
	<i>Escherichia coli</i> SR		<i>Escherichia coli</i> CI		<i>Klebsiella pneumoniae</i>		<i>Pseudomonas aeruginosa</i> SR		<i>Pseudomonas aeruginosa</i> CI	
	Zone of inhibition		Zone of inhibition		Zone of Inhibition		Zone of Inhibition		Zone of Inhibition	
	In mm Mean	As Percentage	In mm Mean	As Percentage	In mm Mean	As Percentage	In mm Mean	As percentage	In mm Mean	As Percentage
Chloramphenicol 30 mcg	18.85±0.23	100	15.25±0.58	100	19.19±0.21	100	21.33±0.57	100	16.98±0.28	100
Fraction II	9.38±0.57	49	7.99±0.21	52	9.18±0.02	47	6.560.15	30	5.55±0.47	32
1/10 dilution of fraction II	8.27±0.33	43	6.33±0.45	41	5.97±0.41	31	5.13±0.98	24	4.77±0.51	28
1/100 dilution of fraction II	4.16±0.14	22	3.26±0.15	21	2.69±0.04	14	3.15±0.21	14	2.17±0.15	37
Ethylene glycol	6.00±0.00	00	6.00±0.00	00	6.00±0.00	00	6.00±0.00	00	6.00±0.00	00

the comparable activity to the standard antibiotics. It was found that cow urine extract (fraction II) showed the highest inhibition in gram-positive *S. aureus* (CI, 213%) and comparable activity in *S. pneumoniae* (95%) compared to standard antibiotics, Chloramphenicol (30 µg), Nalidixic acid (10 µg), Rifampicin (30 µg), and Ampicillin (10 µg) (Table I). In gram-negative bacteria all antibiotics were

inactive, except Chloramphenicol (30 µg), while fraction II showed significant ($p < 0.05$) activity 35% and 37% against *E. coli* (CI) and *K. pneumoniae* (lab strain) as compared to Chloramphenicol (Table II). The results are encouraging, as all other antibiotics were inactive against this strain. The present study suggest that gau-mutra extract from *DSDS* possesses significant ($p < 0.001$) anti-bacterial

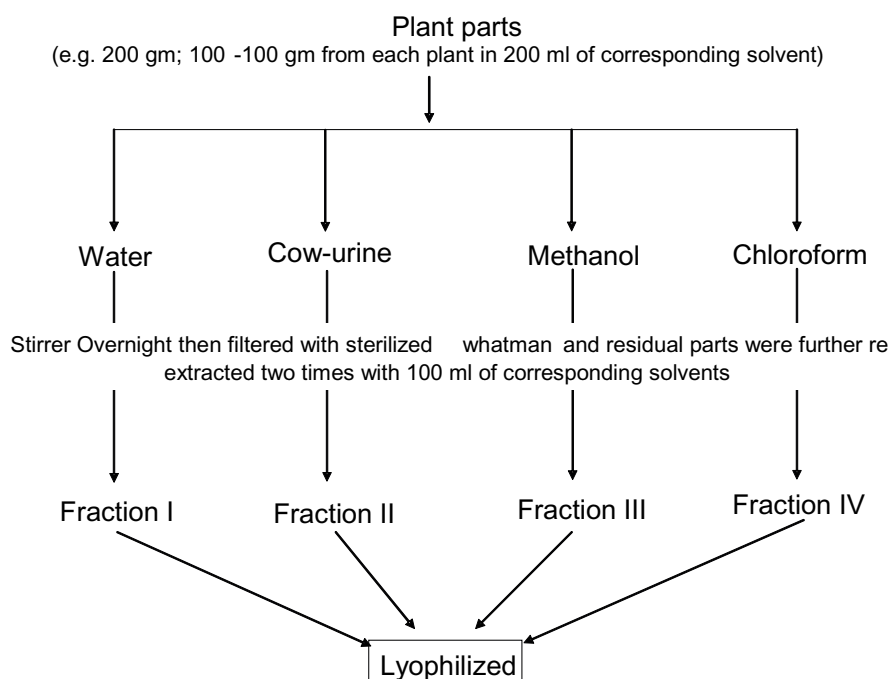


Fig. 1. Preparation of various herbal extracts.

activity compared to standard antibiotics and pure gau-mutra on pathogenic gram-positive *S. aureus* (CI) bacteria. It is also demonstrated that this herbal preparation (DSDS) improved antibacterial activity of gau-mutra extract compared to gau-mutra alone. The clinical isolates which are actually responsible for the invasion and infection, and have developed resistance to the standard antibiotics, were sensitive to this fraction.

DISCUSSION

The increasing prevalence of antibiotic resistance in infectious bacteria, ultimately increasing prevalence of infectious diseases in developed as well as developing countries, has raised the demand for the scientific community to search for new anti-bacterial components (24-25). Natural sources are the best to find new and noble anti-bacterial substances that can help to resolve this problem to some extent (26). In India, as well as various other parts of the world, large plants have been used as effective anti-microbials (27). Therefore, the present

study was designed to evaluate the anti-bacterial potential of cow-urine and two traditional plants: *D. sissoo* and *Datura stramonium*. In this study, we used cow urine as one of the solvents for preparation of a herbal mixture; cow urine is a well-defined solvent in the ancient medical system in India (Ayurveda). Surprisingly, we have observed that cow urine alone has the potential of an anti-bacterial property against both gram-positive and gram-negative. Furthermore, its extract with *D. sissoo* and *Datura stramonium* significantly enhanced the anti-bacterial potential as compared to other solvents (water, methanol and chloroform). Although the anti-bacterial potential was also higher in water extract (fraction I), it was significantly lower than cow urine extract (fraction II). These results indicate that the anti-bacterial activity of these extracts might be due to the presence of anti-microbial phytochemicals i.e. alkaloids, flavinoids, tennins, bioactive peptides, modified aminoacids etc, which are soluble in water, but not soluble in methanol and chloroform (28). Thereby, methanol and chloroform extracts were not able to show any activity against

pathogenic microbial growth. Moreover, the results of the present study also indicate that pure cow urine exhibited good anti-bacterial potential, more than standard antibiotics. It indicates that the cow urine itself has some potent anti-microbial substances. The extraction of DSDS with cow urine increased the efficacy of the particular fraction. The reason behind this may be the synergistic effect of DSDS and cow urine. It will be interesting to carry out further studies for isolation and characterization of active formulas from cow urine alone and from its combination with *Dalbergia sissoo* and *Datura stramonium*. Similarly, various traditional Indian plants have been described as having anti-bacterial properties (29).

Moreover, the results of the present study clearly indicate that Chloramphenicol had higher efficacy against all the selected strains from both gram-positive and gram-negative groups, and similar efficacy is observed in the cow urine extract, which indicates that this fraction had similar effects to Chloramphenicol by inhibiting bacterial growth. However, the anti-bacterial effect of DSDS might be either unidirectional (as explained above) or might be a combined effect of action on various targets i.e. DNA, RNA and protein levels, which needs to be extensively explored further. Furthermore, our preliminary results shown that cow urine has anti-bacterial properties due to the presence of various bioactive peptides that are under process to isolate, characterize and study detailed mechanisms of action.

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