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Immunomodulatory Potential Of Cow Urine

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ABSTRACT

Traditional and folklore medication systems play important role in the maintenance of health around the globe. The rational design of novel drugs from traditional medicines offer new prospects for modern health-care. Cow-urine has been used from ancient times for curing many ailments of human beings. The aim of present study was to evaluate the whole freeze dried cow urine and the fractions of cow urine for their immunomodulatory potential in rats, using Delayed type hypersensitivity reaction, Neutrophil adhesion test, Hematological parameters like RBC, WBC counts and Carbon clearance test for phagocytic index. As per findings freeze dried cow urine exhibited better effect on immune system compared to methanol, ethyl acetate, aqueous and acetone fractions of cow-urine.

Keywords: Cow urine, Cow urine fractions, Immunomodulatory activity.

INTRODUCTION

Ayurveda - an original holistic system of diagnosis and treatment involving nutrition, hygiene and rejuvenation method developed and perfected in India over 5000 years. It is the knowledge of healthy living along with the treatment of illness, if needed [1]. Immunomodulators have great promise in the prevention and treatment of wide range of inflammatory diseases of skin, gut, respiratory tract, joints, and central organs. The functions and efficiency of immune system may be influenced by exogenous factors like food, pharmaceuticals, physical and psychological stress and hormones etc. resulting in immunostimulation or immunosuppression [2].

Urine consisting of water (95%), urea (2.5%) and 2.5% of minerals, salts, hormones and enzymes which have been scientifically proven as safe medication. From ancient times cow-urine (Go-mutra) was commonly used to prevent and cure many diseases with its references in Ayurveda. Apart from its effect in humans it has beneficial role on plant to improve their quality, enhancing the growth and their prevention from insects, pests [3].

Recently cow urine has been reported as bioenhancer of some allopathic antibiotics and anti-cancer drugs. Cow urine not only enhanced their effect but also reduced the toxic and other adverse effect of these synthetic drugs [4]. As per Ayurveda cow urine is needed to purify and detoxify many crude drugs. Panchagavya comprising five cow products including urine has many activities such as immunomodulation and neuropharmacological action [5]. Cow ghee processed with herbals known as “ghrita” has CNS activity [6], immunomodulatory activity [7], hepatoprotective effect [8], wound healing activity [9], sedative and anticonvulsant activity [10] which has been reported on experimental animals. Cow urine distillate known as ‘Kamdhenur ark’ exhibited many biological activities including immunomodulatory potential [11] and antimicrobial effect [12].

Since cow urine is employed for curing many human ailments and therefore it was thought worthy to explore its immunomodulation potential.

MATERIALS AND METHODS

Processing and Fractionation of cow urine:

Healthy virgin cow of around two years age was selected for procuring urine and first (morning) urine collected in a glass bottle was filtered through Whatmann filter paper and then processed for fractionation. The fractions were freeze dried using Freeze drier.

Fractionation of cow urine:

Whatmann filtered Cow-urine was successively fractionated in separating funnel using half of its petroleum ether (60-80°C), ethyl acetate, acetone and methanol as solvent. With each solvent the urine was shaken thoroughly for 15 minutes followed by separation of each solvent layer in different Petri-plates. Finally remaining, Cow-urine in separating funnel was also collected in separate Petri-plate and the whole procedure repeated till sufficient volumes of fractions were collected.

Freeze drying of Fractions:

Each of 250ml fractions so obtained were freeze dried at -64°C for 2 days. In the process the samples were first cooled, and then they were iced. Due to high vacuum in the freeze dryer the iced urine fractions were turned into solid mass. The pet ether fraction of Cow-urine has not yielded any residue indicating the absence of any pet ether soluble matter in Cow-urine. All the samples after freeze-drying were hygroscopic in nature and therefore stored in a well-closed container. Whole cow urine was also freeze dried in the same manner (Table 1).

Sheep red blood cells: SRBCs (Haffkine Biopharmaceuticals Ltd., Mumbai) washed thrice with large volume of pyrogen-free sterile saline and adjusted to a concentration of 5×10^9 cells per ml were used for immunization and challenge.

Experimental Animals;

Studies were carried out on healthy albino rats (120-150g) of either sex securing permission from Institutional Animal Ethical Committee of the Department of Pharmaceutical sciences of Dr. H. S. Gour University Sagar, M.P. The animal were housed under standard condition of temperature ($24 \pm 1^{\circ}\text{C}$), 12/12 h light/dark cycle and fed on standard pellet diet.

Experimental**Treatment**

Albino rats were divided into groups of six animals each. Group I served as control and administered water *ad libitum*. Group II was administered with 500mg kg⁻¹ body weight of whole freeze dried cow urine whereas Group III-VI were administered with 500mg kg⁻¹ body weight ethyl acetate, methanol, acetone and aqueous fractions of cow urine respectively. Each experiment was performed on fresh group of animals and in duplicate.

Delayed Type Hypersensitivity reaction (DTH) using SRBC as an antigen:

The method of Lagrange *et al.* 1974[13] with some modification was followed. Rats of either sex were divided into six groups of six animals each and one group served as control. The rats were primed intraperitoneally with 20µl of suspension containing 5×10⁹ SRBC per ml, on day 0 and challenged on day 21 with 20µl of 5×10⁹ SRBC in right hind foot pad. The left hind foot received an equal volume of saline. The thickness of the foot pad was measured at 0, 4, 24 hrs after challenge using plethysmometer. Freeze dried cow urine and all the fractions of cow urine were administered (500 mg/kg body wt.) once a day for 21 days starting from day zero.

Macrophage Phagocytosis by Carbon Clearance method:

The method of Biozzi *et al.* 1953 [14] was used and rats of either sex were divided into six groups of six animals each. One group served as control. Animal of remaining groups were administered whole freeze dried cow urine and fractions of cow urine a 500mg/kg once a day for 15 days. On day 16, rats were injected with 0.1 ml of carbon suspension (Indian ink) intravenously through tail vein. Blood samples (25 µl) were collected from orbital plexus immediately before and at 5, 10, 15 min interval after the injection of carbon suspension. The blood samples were lysed with 2 ml of 0.1% acetic acid and absorbance was measured spectrophotometrically at 675 nm. The graph of absorbance against time was plotted for each animal in its respective test group and phagocytic index was calculated using the formula:

$$\text{Phagocytic Index (PI)} = \frac{K_{\text{sample}}}{K_{\text{standard}}}$$

Where K_{sample} represents the slope of absorbance versus time curve for blood sample after treatment and K_{standard} represents the slope of absorbance versus time curve for blood sample collected before treatment.

Neutrophil Adhesion Test:

The method described by Wilkonson 1978 [15] was used for evaluating the effect of sample on neutrophil adhesion. Neutrophil adherence was analyzed by the initial Total Leukocyte Count (TLC) and Differential Leukocyte Count (DLC) from the blood sample. After 14 days of treatment of all the six groups, blood samples were collected by retro-orbital puncture in vials containing EDTA as anticoagulant and subjected to total as well as differential leukocyte count. After initial counts, the blood samples were incubated with 80 mg/ml of nylon fibers at 37°C for 15 min. All incubated samples were again analyzed for total and differential leukocyte count. The product of total and percent neutrophil gives Neutrophil Index (NI) of blood samples. Percent neutrophil adhesion was calculated by:

$$\text{Neutrophil adhesion (\%)} = \text{NI}_u - \text{NI}_t / \text{NI}_u \times 100$$

Where NI_u = Neutrophil index of untreated blood samples

NI_t = Neutrophil index of treated blood samples.

Hematological profile:

The method described by Papageorgiou *et al.* 1997 [16] was used. After 25 days of administration of whole freeze dried cow urine and all the fractions of cow urine at the doses of 500mg/kg body weight, blood was collected from the retro-orbital plexus of each animal. The hematological parameters such as Hemoglobin content and Leukocytes, Erythrocytes and Platelet count were determined before and after the dosing.

Statistical Analysis

The results were expressed as mean \pm SEM. All statistical comparisons were made using t- test and p- value less than 0.05 was considered as significant.

RESULTS AND DISCUSSION**Delayed Type Hypersensitivity reaction (DTH) using SRBC as an antigen:**

In the present investigation, SRBC- induced delayed type hypersensitivity used to assess the effect of freeze dried cow urine and all its fractions on cell mediated immunity revealed that the DTH reaction in group I (control) and group (II-VI) was more severe at 24 hour. The maximum increase in skin thickness was compared at 24 hour with the control. The results for control, whole freeze dried cow urine and ethyl acetate, methanol, acetone and aqueous fractions were 0.34 ± 0.03 , 0.47 ± 0.05 , 0.38 ± 0.03 , 0.40 ± 0.03 , 0.35 ± 0.03 , 0.36 ± 0.05 respectively. The mean increase in thickness of skin and tissue reaction produced by SRBC in rats suggested enhancement of CMI response on regular use of freeze dried cow urine (Table 2).

Determination of Macrophage Phagocytosis by Carbon Clearance method:

In the present study an increased phagocytic activity was observed in treated groups as compared to control. The rate of carbon clearance was determined from phagocytic index. It was found to be 1.55 ± 0.51 for freeze dried cow urine which suggests its effect on Phagocytosis. The fractions has the phagocytic index of about 1.52 ± 0.42 , 1.51 ± 0.41 , 1.48 ± 0.39 , 1.45 ± 0.36 for methanol, ethyl acetate, aqueous and acetone fractions of cow urine respectively. The percentage increase in phagocytic index of freeze dried cow urine, methanol, ethyl acetate, aqueous and acetone fraction was nearly 55%, 52%, 51%, 48% and 45% respectively (Table 2).

Neutrophil Adhesion Test

The percent neutrophil adhesion in control group animals was found to be 23.34 ± 2.1 and for whole freeze dried cow urine it was 27.32 ± 1.8 while for the fractions of cow urine i.e. methanol, ethyl acetate, aqueous and acetone fractions the values were 25.46 ± 1.6 , 24.55 ± 1.4 , 24.20 ± 1.2 , 22.79 ± 1.1 respectively. As it is evident from the results of neutrophil adhesion test, significant increase (16%) in neutrophil adhesion was observed in whole freeze dried cow urine, however increase in percentage of neutrophil adhesion test was comparatively very less 9% to 4% with fractions of cow urine. (Table 3)

Hematological profile:

The results of hematological parameters are given in Table (4 & 5). The effect of freeze dried cow urine and all the fractions of cow urine on hematological parameters were studied before and after 25 day of treatment on rats. The parameters like hemoglobin content, red blood cells count, white blood cell count and platelet count were affected by all cow urine samples. The ethyl acetate fraction markedly reduced the RBC count by nearly 10% and increased the WBC count by 17% in the rats whereas for acetone fraction RBC count was decrease nearly by 8% and WBC count increased by 14%. On comparing the variation in RBC count, the intensity of

action of ethyl acetate fraction was higher with respect to the acetone fraction i.e. the rats administered with the ethyl acetate fraction produced severe fall in Red blood cell count. Comparing the variation in WBC count, the intensity of action of ethyl acetate fraction was also more than acetone fraction. The result proves that these two fractions of cow urine have the potential to boost the immune functions by increasing the WBC counts and subsequently reducing the RBC count to certain extent. Other fractions and freeze dried cow urine showed positive increase in hematological parameters. The results suggested that dried cow urine and its methanol and aqueous fractions increased the hemoglobin content nearly by 8%, 7% and 6% respectively. Freeze dried cow urine and its methanol and aqueous fractions increased the RBC count. The result of RBC count suggest the values for freeze dried cow urine (10%) and methanol and aqueous fractions (9%). The result of WBC count suggest an increase in number of cells after 25 days. It was nearly 13% for freeze dried cow urine, and for methanol and aqueous fractions it was nearly 12% and 8% respectively. The results of platelet count suggested an increase in its number by nearly 6-8% in all the samples.

Immune activation is an effective protective approach against emerging infectious diseases. Control of diseases by immunological means is through the development and improvement of protective immunity by the avoidance of undesirable immunological side reactions like myelosuppression using of various immunosuppressive agents. The whole freeze dried cow urine and its different fractions have been evaluated at an oral dose of 500 mg/kg body weight. Cow urine has been reported with beneficial properties for the cure of human beings and thus regular use of fresh morning cow urine may make the person free from many diseases.

Table 1: Characteristics of freeze dried cow urine and its fractions

S. No.	Parameter	Cow urine fractions				
		Whole cow urine	Ethyl acetate	Acetone	Methanol	Aqueous
01.	Color	Light Yellowish orange	Dark Brown	Dark Brown	Light to Dark Brown	Light Yellowish Orange
02.	Odor	Pungent	Pungent	Pungent	Pungent	Pungent
03.	Taste	Bitter, Sweet sour	Bitter, Sweet Sour	Bitter, Sweet, Sour	Bitter, Sweet, Sour	Bitter, Sweet, Sour
04.	Solubility	Water, Methanol	Ethyl acetate, Water	Acetone, Water	Methanol, Water	Water
05.	Yield	2.45%	2.20%	2.10%	2.05%	2.06%

Table 2: Effect of whole freeze dried cow urine and its fraction on DTH response and Macrophage Phagocytosis by Carbon Clearance method

Group	DTH response (% increased in foot pad thickness)	Macrophage Phagocytic Index
Control group I	0.34±0.03	1
Test group II	0.47±0.05 (38%) ***	1.55±0.57***
Test group III	0.36±0.03 ^{ns}	1.51±0.42***
Test group VI	0.40±0.03 (18%) ***	1.52±0.41***
Test group V	0.35±0.03 ^{ns}	1.45±0.39*
Test group VI	0.36±0.05 ^{ns}	1.48±0.36*

Statistical analysis was done by one way ANOVA followed by Dunnett's multiple comparison test significant at ** $P < 0.01$, *** $P < 0.001$ as compared to control. Values are expressed in mean±SEM for six observations.

Control group-I – No treatment,

Test group II - Whole freeze dried cow urine (WFCU)

Test group III- Ethyl acetate fraction of cow urine (EtFCU)

Test group VI -Methanolic fraction of cow urine (MFCU)

Test group V - Acetone fraction of cow urine (AcFCU)

Test group VI- Aqueous fraction of cow urine (AFCU)

Table 3: Effect of whole freeze dried cow urine and its fractions on neutrophil adhesion in rats.

Group	TLC ($10^3/\text{mm}^3$) (A)		% Neutrophil (B)		Neutrophil index (A×B)		% Neutrophil Adhesion
	UnB	FTB	UnB	FTB	UnB	FTB	
I Control	6.3±2.9	5.8±2.3	44±2.24	37±3.20	284.9±35.20	214.4±24.3	23.34±2.1
II WFCU	6.8±1.4	6.1±1.6	55±2.20	44±1.37	362.2±32.31	263.2±31.5	27.33±1.8***
III EtFCU	6.6±1.2	6.0±1.6	53±2.23	46±1.59	350.3±31.51	276.2±33.4	24.60±1.4*
IV MFCU	6.7±1.2	6.1±1.4	54±2.21	45±1.47	356.2±31.42	274.5±32.2	25.46±1.6**
V AcFCU	6.6±1.2	5.9±1.3	51±2.24	49±1.40	344.2±34.42	289.2±36.3	22.79±1.0*
VI AFCU	6.5±1.4	6.0±1.5	52±2.21	47±1.35	346.5±31.62	282.3±34.6	24.20±1.2*

UnB-untreated blood

FTB-nylon fiber treated blood

Statistical analysis was done by one way ANOVA followed by Dunnett's multiple comparison tests. * $P < 0.5$ - not significant, ** $P < 0.25$ Significant, *** $P < 0.05$ considered higher significant. Values are expressed in mean±SEM for six observations.

WFCU- Whole freeze dried cow urine

EtFCU- Ethyl acetate fraction of cow urine

MFCU- Methanolic fraction of cow urine

AcFCU- Acetone fraction of cow urine

AFCU- Aqueous fraction of cow urine

Table 4: Effect of whole freeze dried cow urine and its fractions urine on Hematological profile.

Group	Hemoglobin Concentration (g %)		Red Blood Cells (million/cmm)	
	0 day	25 day	0 day	25 day
Control	11.9±0.22	11.7±0.24	8.60±0.23	8.60±0.23
II WFCU	11.8±0.21	12.7±0.22	8.62±0.21	9.44±0.24
III EtFCU	11.7±0.22	10.5±0.25	8.63±0.21	7.70±0.24
IV MFCU	11.8±0.20	12.6±0.23	8.62±0.23	9.41±0.24
V AcFCU	11.7±0.21	10.8±0.24	8.63±0.22	7.74±0.24
VI AFCU	11.9±0.20	12.5±0.21	8.620.24	9.40±0.21

WFCU- Whole freeze dried cow urine

EtFCU- Ethyl acetate fraction of cow urine

MFCU- Methanolic fraction of cow urine

AcFCU- Acetone fraction of cow urine

AFCU- Aqueous fraction of cow urine

Table 5: Effect of whole freeze dried cow urine and its fractions on White blood cells and Platelet Count.

Group	White blood cells (thousand/ cmm)		Platelet count (thousand /cmm)	
	0 day	25 day	0 day	25 day
I Control	6.80±0.81	6.82±0.45	540.2±0.22	541.3±0.23
II WFCU	6.91±0.80	7.83±0.26	542.3±0.21	584.0±0.21
III EtFCU	6.73±0.83	7.90±0.24	540.3±0.41	580.4±0.23
IV MFCU	6.92±0.80	7.72±0.27	541.4±0.20	583.2±0.22
V AcFCU	6.90±0.82	7.86±0.25	540.5±0.24	572.6±0.24
VI AFCU	6.91±0.82	7.49±0.25	42.4±0.23	579.3±0.21

WFCU- Whole freeze dried cow urine

EtFCU- Ethyl acetate fraction of cow urine

MFCU- Methanolic fraction of cow urine

AcFCU- Acetone fraction of cow urine

AFCU- Aqueous fraction of cow urine

SUMMARY AND CONCLUSION

The use of herbs and minerals for improving the overall resistance of body against common infections and pathogens has been a guiding principal of Ayurveda [18] and therefore the composition of cow urine with its mineral content may be responsible for its immunomodulatory activity.

Whole cow urine and its fractions showed good immunomodulatory potential and hence it can be suggested that the freeze dried cow urine and its fractions can improve the over all non-specific parameters of immunity. Although ethyl acetate and aqueous fractions boost the immune system and acetone fraction of cow urine has also shown some effect on immunomodulation, freeze dried cow urine and its methanol fraction has shown better immunomodulation potential in experimental models.

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