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### RESEARCH ARTICLE

#### DETECTION OF PHYTOCHEMICALS IN COW URINE AND THEIR ROLE AS ANTIMUTAGENS.

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#### Abstract

Cow urine is used for various ailments by Ayurvedic and Unani pharmacy and also by traditional healers. This study was aimed at carrying out phytochemical analysis and antimutagenic activity of Cow urine. Phytochemical screening revealed the presence of alkaloids, carbohydrates, proteins, steroids, terpenoids and cardioglycosides. Phenolic flavonoids, phlobatannins, anthrocyanins, anthraquinone, tannins and saponins were totally absent. Cow urine is an important source of substances claimed to induce antimutagenic effect. Cow urine works as an antimutagenic agent due to the antioxidant property of uric acid and allantoin present in it. Cow urine has been granted U.S. patents (No. 6, 896, 907 and 6, 410, 059) for its medicinal properties and it functioning as an anticarcinogenic agent. Cow urine has been observed to increase the potency of "Taxol" (paclitaxel) against MCF-7, a human breast cancer cell-line in invitro assays (U.S. patent No. 6, 410, 059). Ames test is used for the study of the antimutagenic effect of cow urine when used in combination against sodium azide, 2-nitrofluorine and mitomycin C in absence of metabolic inhibitor (Sq). Combinations of varying concentrations were checked and the percentage of inhibition was determined using the formula:  $[(a-b)/(a-c)] \times 100$ . The combination showed a gradual respective decrease in the number of revertant colonies against tester strains Salmonella typhimurium TA 100, TA 1535, TA 98 and TA 102. The percentage inhibition ranged from 5% to 85%.

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#### Introduction:-

Indian Ayurvedic doctors regularly use cow urine as a natural medicine to treat many common disorders<sup>1</sup>. Very few attempts have been made to correlate scientifically the nature of cow urine. In order to correlate ancient and traditional knowledge of use of cow urine with the scientific methodologies and parameters of modern times, this study has been done using cow urine in different forms as immunomodulatory and antioxidant agent<sup>4,6</sup>.

In Sushrita Samhita, cow urine has been described as an extremely effective substance or material secretion of animal origin having a wide and innumerable therapeutic value. In India, oral consumption of cow urine has been prescribed and practiced since ages. Effect of cow urine along with a combination of medicinal plants, has been found to enhance its effectiveness in the cure of diabetes, cancer, and AIDS<sup>12</sup>. This type of an alternative and combo treatment is referred to as "Cowpathy".

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Traditional medicines have recommended direct consumption of cow urine as an effective medicine which is easily available and very easily affordable. It contains twenty four types of salts, phenol, potash, iron, calcium, phosphorus and lactose. Phenol, being the major material constituent of cow urine exhibits antiseptic activity. It is a mixture of phenol and cresol.

Cow urine has also been observed to lead to the increase in potency of "Taxol" (pactitaxel) against MCF -7, a human breast cancer cell line "invitro assays" (U.S. Patent Numbers 6, 059, 410). These significant achievements highlights the potential role of cow urine in the treatment of various bacterial infections and also cancer, thus proving that cow urine plays a prominent role in enhancing the efficacy and potency of other drugs.

The objective of the present study is to make a deep search into the bio enhancing property and the impact of the naturally available products, so that they can be used to combat many common diseases. Bio enhancing is one of the many properties of cow urine. It promotes and increases the effectiveness of antimicrobial, antifungal, and anticarcinogenic drugs. It also leads to an increase in the activity of gonadotropin releasing hormone conjugate with bovine serum albumin and zinc<sup>3</sup>.

Phytochemical analysis of cow urine has also shown the presence of tannins and phlobatannins, which are responsible for antimutagenic activity<sup>9</sup>. Today, bacteria are being used for the assessment of antimutagenic activities of these different compounds in a short time with excellent results. One of the methods used for assessing the mutation prevention properties of a compound in bacteria is the Ames test<sup>4,5</sup>.

Ames test is a worldwide short term bacterial reverse mutation test. It is known so because new mutation at the site of the pre existing mutation can restore the gene function and allow the cells to synthesize Histidine. The test is designed for screening a variety of new chemical substances and drugs that can produce genetic damage that leads to gene mutation<sup>8,11</sup>. The *Salmonella typhimurium* strains used in the test have different mutations in various genes in the Histidine operon, and each of these mutations are designed to be responsive to mutagenic agents via different mechanisms<sup>11,13</sup>.

## Materials and Methods:-

### Procurement of Cow Urine:-

Fresh cow urine has been procured from pure Indian breed Junagadh Gir cow based at the Goshala of Gurukulsupa near Navsari for use in this study. Fresh urine collected was filtered and centrifuged in order to remove all the debris and precipitated materials. Cow urine is then sterilized by autoclaving it at 121 °C and 15 lb/sq inch pressure for 20 minutes. It is then preserved at 4 °C for further long time use<sup>12</sup>. In order to assure that the cow urine is free of microbes, it is inoculated in broth and also on nutrient agar plate, and sterility is tested.

### Phytochemical Screening:-

Sterile cow urine was subjected to standard procedures for carrying out different qualitative chemical tests in order to recognize the constituents<sup>2,7,14,15,16</sup>.

### Procedure Adopted for Qualitative Analysis of the Phytochemical Constituents:-

<b>Alkaloids</b>	1 ml of the filtrate on mixing with 2 ml of Dragendroff's reagent shows a development of turbid orange colour.
<b>Tannins</b>	1 ml of the filtrate on mixing with 2 ml of Ferric chloride promotes a development of dark green colour.
<b>Saponins</b>	1 ml of the filtrate is added to 2 ml of distilled water and shaken vigorously. It is then allowed to stand for 10 minutes. Foam formation on the top layer of the mixture, which remains for 9 to 11 minutes is an indication of the presence of saponins.
<b>Anthraquinones</b>	1 ml of the filtrate is mixed with 10 ml of benzene, and is then filtered. 5 ml of 10% ammonia (v/v) is then added to the above filtrate and shaken vigorously. Development of pink tone in the solution points the presence of anthraquinones.
<b>Anthrocyanides</b>	1 ml of the filtrate when mixed with 5 ml of dilute HCl gives pale pink tone.
<b>Phenolic flavonoids</b>	1 ml of the filtrate when mixed with 2 ml of 10% lead acetate leads to the formation of brown precipitates.
<b>Flavonoids</b>	1 ml of the filtrate on mixing with 2 ml of dilute sodium hydroxide shows the development of golden yellow tone.

<b>Carbohydrates</b>	<ol style="list-style-type: none"> <li>1 ml of the filtrate is mixed together with 5 ml of Benedict's reagent and boiled for 5-6 minutes. Formation a bluish green colour, which shows the presence of carbohydrates.</li> <li>Addition of a 2-3 drops of Molisch's reagent and 2-3 drops of concentrated H<sub>2</sub>SO<sub>4</sub> to 1 ml of the filtrate, gives a purple colour.</li> <li>Addition of few drops of Fehling's A reagent to 1 ml of the filtrate, leads to the development of green colour.</li> <li>Addition of few drops of Fehling's B reagent to 1 ml of the filtrate, leads to the development of brown colour</li> </ol>
<b>Proteins</b>	Addition of 5 to 6 drops of Million's reagent to 1 ml of the filtrate, leads to the formation of white precipitates, turning red on heating.
<b>Steroids</b>	1 ml of chloroform and 1 ml of H <sub>2</sub> SO <sub>4</sub> are added slowly by the sides of the test tube containing 1 ml of the filtrate. If the upper layer turns red in tone and the sulphuric acid layer exhibits a greenish yellow fluorescence, then steroids are present.
<b>Terpenoids</b>	Addition of 2 ml chloroform and a 2-3 drops of concentrated sulphuric acid carefully to 1 ml of the filtrate leads to the development of reddish brown colour at the interface.
<b>Cardiac Glycosides</b>	Addition of 1 ml of FeCl <sub>3</sub> reagent (consisting of 1 volume of 5% FeCl <sub>3</sub> solution and 99 volume of Glacial acetic acid) and a few drops of concentrated sulphuric acid, greenish blue colour appears within few minutes.
<b>Phlobatannins</b>	Addition of few drops of 1% HCl to 1 ml of the filtrate leads to the formation of red precipitates.

#### List of Tester Strains and Positive Controls as Mutagenic Agents:-

<b>TA100</b>	Sodium azide	5.0 µg / Plate
<b>TA98</b>	2-Nitrofluorine	7.5 µg / Plate
<b>TA102</b>	Mitomycin C	0.5 µg / Plate
<b>TA1535</b>	Sodium azide	0.5 µg / Plate
<b>TA1537</b>	9- Aminoacridine hydrochloride hydrate	75.0 µg / Plate

#### Anti Mutagenicity Test:-

- Two tubes, each containing 2 ml of top agar with histidine and biotin (0.5 mM), previously melted and maintained at 45 ± 2 °C was used for plating.
- A volume of 0.5 ml of 0.2 M phosphate buffer was added to each tube in the absence of S9 mix.
- In one set, 100 µL of different concentrations of mixture of cow urine and plant extract were added to the above mixture.
- In the second set, 100 µL of different concentrations of cow urine and relevant mutagen for each strain were added to the above serial no. 1 and 2 mixtures. The concentrations of the test sample for investigating the antimutagenicity were: 25, 50 and 100 µL / Plate. These were tested against sodium azide (5.0 µg / Plate) in TA 100 and TA 1535; 2-Nitrofluorine (7.5 µg / Plate) in TA 98; Mitomycin C (0.5 µg / Plate) in TA 102; and, 9-Aminoacridine Hydrochloride Hydrate (75.0 µg / Plate) in TA 1537.
- For the negative control, 100 µL of sterile distilled water was added.
- For the positive control, 100 µL of relevant mutagen were added for each tester strain.
- Finally, 100 µL of overnight grown standard bacterial culture were added to all the top agar tubes.
- The mixture was added to all the top agar tubes.
- All the tubes were kept in a water bath maintained at 37 °C for 30 minutes.
- The contents were mixed thoroughly and rapidly transferred to the individual petri dishes containing Minimal Glucose Agar.
- After solidification of the contents, the petri dishes were inverted and incubated at 37±1 °C for 48 - 72 hours and then assessed by scoring the number of revertant colonies of individual plates<sup>5,10</sup>.

#### Results and Discussion:-

**Table 01:-** Phytochemical Analysis of Cow Urine Sample.

Detection test for	Cow Urine Sample	
	Present ●	Absent ○
Alkaloids: Dragendorff's Test	●	
Tannins		○

Saponins		○
Anthraquinone		○
Anthrocyanosid		○
Phenolic flavonoids	●	
Flavonoids		○
Carbohydrates: Benedicts test	●	
Proteins	●	
Steroids	●	
Terpenoids	●	
Cardiac glycosides	●	
Phlobatannins		○

Table 01 shows the phytochemical analysis of cow urine to indicate the presence of alkaloids, phenolic flavanoids, steroids, terpenoids, cardiacglycosides, carbohydrates and proteins. They are all responsible for antimicrobial activity invitro and invivo.

#### Anti Mutagenecity Test:-

Cow urine was tested for their antimutagenic activity against sodium azide, 2-nitrofluorine, 9-aminoacridine hydrochloride hydrate and mitomycin C induced mutagenicity in the tester stains TA100 and TA1535, TA98, TA1537 and TA102 respectively. Varying effects on cow urine on the direct acting mutagens induced mutagenicity in one or more tester strains are presented in the Table 02. The inhibitory activity was expressed as percentage decrease of reverse mutation.

$$\% \text{ inhibition} = [(a-b) / (a-c)] \times 100$$

Where,

- a » Number of histidine revertants induced by mutagen
- b » Number of histidine revertants induced by mutagen in the presence of cow urine
- c » Number of revertant induced in negative control

**Table 02:-** Number of Revertant Colonies.

Concentrations (µl/plate)	His <sup>+</sup> Revertant Colonies/Plate (Absence of Metabolic Activation)									
	TA1537		TA1535		TA98		TA100		TA102	
	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
NC	1	4	16	13	21	21	120	132	215	229
NC + CU	2	4	14	13	18	19	132	128	191	212
PC	287	263	160	170	485	425	670	685	985	1112
PC + HD	280	243	75	68	197	208	101	97	208	316
PC + MD	276	232	110	90	260	255	160	156	301	418
PC + LD	279	240	112	102	390	395	420	417	984	990

#### Notations in Table 02:-

NC	Sterile Distilled Water, 100 µL / Plate
NC + CU	Sterile Distilled Water, 100 µL / Plate + Cow Urine, 100 µL / Plate
PC	For TA1537: 9- Aminoacridine hydrochloride hydrate, 75.0 µg / Plate, For TA1535: Sodium Azide, 0.5 µg / Plate For TA98: 2-Nitrofluorine, 7.5 µg / Plate For TA100: Sodium Azide, 5.0 µg / Plate For TA102: Mitomycin C, 0.5 µg / Plate
HD	100 µL Cow Urine / Plate
MD	50 µL Cow Urine / Plate
LD	25 µL Cow Urine / Plate

**Table 03:-** Percentage Reduction in Number of Revertant Colonies.

Concentration μL / Plate	His <sup>+</sup> Revertant Colonies/Plate (Absence of Metabolic Activation)				
	TA1537	TA1535	TA98	TA100	TA102
PC	0.0	0.0	0.00	0.0	0.00
HD	5.0	56.55	55.2	85.35	75.25
MD	7.8	39.1	43.2	76.65	65.9
LD	5.75	35.0	13.3	38.2	5.55

**Notations in Table 03:-**

PC	For TA1537: 9- Aminoacridine hydrochloride hydrate, 75.0 μg / Plate For TA1535: Sodium azide, 0.5 μg / Plate For TA98: 2-Nitrofluorine, 7.5 μg / Plate For TA100: Sodium azide, 5.0 μg / Plate For TA102: Mitomycin C, 0.5 μg / Plate
HD	100 μL Cow Urine / Plate
MD	50 μL Cow Urine / Plate
LD	25 μL Cow Urine / Plate

**Conclusion:-**

- Cow urine exhibited nearly 50% decrease in the number of revertants colonies against sodium azide, 2-nitrofluorine and mitomycin C induced mutagenicity in the tester stains TA98, and TA1535 whereas there was more than 75% decrease in tester strain TA100 and TA102 at high concentration of cow urine of 100 μL / Plate.
- At 50 μL / Plate concentration of cow urine, percent inhibition of mutagenicity against sodium azide, 2-nitrofluorine and mitomycin C ranged from 7.8% to 76.65%. (Table.2) Tester strain TA102 and TA100 the percentage decrease was from 65% to 77%. whereas the rest of the strains showed moderate results.
- At 25 μL / Plate concentration of cow urine, percentage inhibition of mutagenicity against sodium azide, 2-nitrofluorine and mitomycin C was unremarkable. Hence, it can be concluded that Antimutagenic activity of the cow urine is concentration dependent.
- The presence of phytochemicals like alkaloids, phenolic flavanoids, steroids, terpenoids, cardiacglycosides, carbohydrates and proteins are accounted to be responsible for their antimutagenic nature.

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