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In vitro studies on antifungal activity of cow urine distillate against fungi causing opportunistic infections

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ABSTRACT

Cow has been considered as a sacred animal in our country. Cow urine is placed in a unique position in Indian system of medicine. In the present investigation, Cow urine distillate obtained from local shops has been subjected to antifungal activity against three test fungi namely *Aspergillus niger, Aspergillus oryzae* and *Mucor* sp. which cause opportunistic mycotic infections. The antifungal activity was assessed using Poison food technique and Spore germination inhibitory activity. Considerable reduction in fungal growth, in terms of reduced colony diameter when compared to control, was observed in trials. Extent of sporulation was also reduced in plates poisoned with Cow urine distillate. The percentage of germination of spores and germ tube length was also reduced significantly. The activity of Cow urine distillate against test fungi was found to be concentration (dose) dependent i.e., higher activity was observed when concentration is increased. The results show that Cow urine distillate possesses active principles responsible for antifungal activity and the use of Cow urine distillate could confer protection against opportunistic mycotic infections.

KEYWORDS

Cow urine distillate;
Antifungal activity;
Poison food technique;
Spore germination inhibitory activity;
Test fungi.

INTRODUCTION

In India, cow is worshipped as "Kamadhenu", the god who fulfills all the desired ones, since thousands of years. All the products obtained from cow are useful to mankind in one or the other way, either spiritually or medicinally. The urine, milk, ghee, curd and dung obtained from cow are together called "Panchagavya" (Five important products of cow) which are having immense importance in spiritual and medicinal aspects in India. All these five are having medicinal properties and are used either singly or in combination with either herbs/minerals against many diseases. This kind of treatment using Panchagavya is termed Panchagavya therapy or Cowpathy which have proved effective in treatment of dreadful diseases like AIDS and Cancer. The urine of cow is known for causing weight loss, reversal of certain cardiac and renal pathologies, curing indigestion, oedema etc. Ayurvedic practitioners routinely use cow urine as a remedy for many diseases and is beneficial in

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diseases like flu, allergies, colds, rheumatoid arthritis, bacterial/viral infections, tuberculosis, chicken pox, hepatitis, leucorrhoea, leprosy, ulcer, heart disease, asthma, skin infections, aging, chemical intoxication etc. In India, drinking of cow urine has been practiced for thousands of years. One of the forms of cow urine with popular use nowadays is Cow urine distillate, a transparent liquid obtained when cow urine is subjected to distillation. To make Cow urine distillate(for oral intake) cow urine is boiled and the vapor is collected (Process is called distillation). The distillate is having more acceptability than crude cow urine and shows activities of crude cow urine. US patent 6,410,059 has been granted to Indian scientists Khanuja *et al.* June 25, 2002 for Pharmaceutical composition containing Cow urine distillate and an antibiotic where Cow urine distillate acts as a bioenhancer of antibiotic activity.

The healing properties of cow urine are mentioned in many ancient Hindu texts and are studied by several authors. There have been several reports saying the importance of cow urine in the prevention of diseases caused by microbes and other physiological factors. The present study is undertaken to find out efficacy of Cow urine distillate against test fungi.

MATERIALS AND METHODS

Cow urine distillate (CUD)

Cow urine distillate was purchased from the local shops of Shivamogga city. The distillate was manufactured by Sri Ramachandrapura Mutt, Hosanagara (Tq), Shivamogga (Dt) using urine of indigenous cow varieties.

Screening for Antifungal activity (Poison food technique and Spore germination inhibition assay)

1. Test fungi

In the present study, we have selected two species of the genus *Aspergillus* (namely *Aspergillus niger, Aspergillus oryzae) and Mucor sp.* Some species of the genus *Aspergillus* are known to cause called Aspergillosis and Mucor sp. causes Mucorosis, the opportunistic mycotic infections. Some species of the genera of fungi tested are also considered to be common laboratory contaminants and potent agents of destruction of stored food and grains.

The pure cultures of the fungi were obtained from the culture collection deposited in the department. The pure cultures of fungi were maintained on Sabouraud's dextrose agar slants. The cultures were maintained in refrigerator for use and regularly checked for contamination. Periodic transfers were made aseptically.

2. Composition and preparation of Sabouraud Dextrose Agar (SDA)

Peptone-10g; Dextrose- 40g; Agar- 20g; Distilled water- 1000ml

The ingredients were carefully weighed, dissolved in measured amount of distilled water and the reaction of the medium was adjusted to 5.6 using dilute acid and alkali (0.1N HCl and 0.1N NaOH). The container was properly plugged and sterilized by autoclaving.

3. Preparation of spore suspension

The fungal inoculum was prepared by making the suspension of the spores of the test fungi in a test tube containing 0.85% sterile normal saline containing 0.05% Tween 80 detergent. The fungal spores were taken from well grown cultures of test fungi using sterile inoculation loop under aseptic conditions and placed in sterile saline solution and mixed well using vortex mixer. The fungal spore suspension was used for inoculation on plates poisoned with the Cow urine distillate.

4. Poison food technique

The principle involved in this technique is to poison the nutrient medium with the substance which is Cow urine distillate and plant extracts in this experiment. The test fungus is allowed to grow on such a poisoned plate. If the test sample is a poison for the fungus then there will be reduction in colony diameter when compared to control (not having any test material). The effect of test samples on fungal growth can be determined by measuring the diameter of the colony obtained on poisoned plate. Generally the result varies with the concentration of the sample being used.

S.D.A medium was prepared, poisoned with Cow urine distillate (5%, 10%, 15% and 20%), sterilized, cooled to about 45°C and dispensed into sterile Petri plates. After solidification of the medium, the test fungi were inoculated by point inoculation method under aseptic condition. The spore suspension of test fungi were taken with the help of sterile inoculation needle and inoculated by touching the centre of the medium without dispersing the inoculum. The plate was incubated at room temperature for 3days. After incubation, the colony di-

ameter was measured and extent of sporulation was recorded.

5. Spore germination inhibition assay

This is a simpler technique which determines the effectiveness of a substance against germination of fungal spores. In this method, the spore suspension along with the substance to be tested was taken in a cavity slide and kept in moist chamber for 24 hours. The formation of germ tube (spore germination) was determined for a minimum of 200 spores and the antifungal activity was interpreted. The parameter considered here was the percentage of spore germination. The spore germination inhibition activity was done as below.

A moist chamber was prepared using Petri dish, inner surface of which, being covered with filter paper. The filter paper was moistened with water in such a way that it is enough to keep the inside of the plate cool. Excess water was removed. The spore suspension of the test fungus was prepared in a test tube containing a little Tween 80, a detergent (so as to mix the spores properly). In a cavity slide two drops of Cow urine distillate was added to about 2 loopful of spore suspension and gently mixed. A control was prepared by adding 2 loopful spore suspension to two drops of distilled water. The slide was placed in a moist chamber for 2 days at room temperature. After incubation in moist chamber, the slides were taken out, observed under microscope for germination of spores.

Percentage of inhibition was calculated by using the formula

% Inhibition = Control-Test/Control×100

RESULTS AND DISCUSSION

Cow urine distillate at various concentrations was tested for antifungal activity. The growth reduction in percentage was taken into consideration and antifungal effect was evaluated. TABLE 1, 2 reveal reduction in vegetative growth of test fungi in poisoned plates. 5%

TABLE 1: Antifungal activity of different concentrations of Cow urine distillate Cow urine distillate concentration in medium: 5, 10, 15 and 20ml out of 100ml

Test fungus	Control		5% CUD		10% CUD		15% CUD		20% CUD	
	C.D	Sp	C.D	Sp	C.D	Sp	C.D	Sp	C.D	Sp
A. niger	5.5	++ ++	5.2	++ ++	4.4	++ +	3.8	++	1.0	+
A.oryzae	3.9	+++	3.5	+	2.5	++	1.9	++	1.5	+
Mucor sp.	7.0	+++ ++	4.4	++ ++	2.3	++	1.7	+	-	-

C.D-Colony diameter; Sp- Extent of sporulation

TABLE 2: Percent growth inhibition of test fungi at different concentrations of Cow urine distillate

Test fungus	% growth inhibition at various Cow urine distillate concentrations			
	5%	10%	15%	20%
A.niger	5.4	20.0	30.9	81.8
A.oryzae	10.2	35.9	51.3	61.5
Mucor sp.	37.1	67.1	75.7	100.0

Cow urine distillate was more effective against *Mucor* sp. (37.1%) followed by *A.oryzae* (10.2%) and *A.niger* (5.4%). Extent of sporulation was reduced to little extent in case of *A.oryzae* and *Mucor* sp. while remains the same in case of *A.niger*. Cow urine distillate at 10% concentration caused growth retardation of 20%, 35.9% and 67.1% in *A.niger*, *A.oryzae* and *Mucor* sp. respectively. Extent of sporulation in fungi was also reduced at 10% concentration. The growth retardation of *A.niger*, *A.oryzae* and *Mucor* sp. was 30.9%, 51.3% and 75.7% respectively at 15% concentration of Cow urine distillate. Sporulation extent has come down drastically in *Mucor* sp. than *A.niger* and *A.oryzae*. Maximum inhibition of test fungi was observed at Cow urine distillate concentration of 20%. The percent growth inhibition of *A.niger*, *A.oryzae* and *Mucor* sp. was found to be 81.8, 61.5 and 100.0 respectively. *Mucor* sp. was completely inhibited. Over 50% growth retardation was observed in *A.niger* and *A.oryzae* among which *A.niger* was inhibited to larger extent. Extent of sporulation of test fungi was drastically reduced. The results obtained revealed concentration dependent or

TABLE 3: Percentage germination of fungal spores in control and in Cow urine distillate and percent inhibition of spore germination in Cow urine distillate when compared to control

Test fungi	Control (Distilled water)			Test (Cow urine distillate)			% inhibition (compared to control)
	Spores counted	No. of spores germinated	% germination	Spores counted	No. of spores germinated	% germination	
Aspergillus niger	200	155	77.5	200	77	38.5	50.32
Aspergillus flavus	200	185	92.5	200	95	47.5	48.64
Mucor sp.	200	169	84.5	200	74	37.0	56.21

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dose dependednt activity of Cow urine distillate against fungi tested. *Mucor* sp. was the most affected fungus when compared to *A.niger* and *A.oryzae*.

The result of spore germination inhibition assay carried out to assess the ability of Cow urine distillate to affect germination of spores of test fungi is depicted in TABLE 3. The number of spores germinated out of 200 spores counted was recorded. The percentage germination of spores was higher in distilled water when compared to germination of spores in Cow urine distillate. *Aspergillus flavus* spores were germinated to about 90% in distilled water while the germination percentage in Cow urine distillate was lesser (48.64%). Same kind of results were obtained with other fungi i.e., germination percent being higher in distilled water (control) when compared to test (Cow urine distillate). Among fungi tested, *Mucor sp.* (56.21%) was inhibited to more extent followed by *A.niger* (50.32%) and *A.flavus* (48.64%). Another important observation being made was that the length of germ tubes was higher in control than the test. On an average, the length of germ tubes in control slides (distilled water) were twice or more when compared to test slides(Cow urine distillate).

There are several reports revealing biological activities of cow urine and its products. Cow urine has a unique place in Ayurveda and has been described in 'Sushrita Samhita' and 'Ashtanga Sangraha' to be the most effective substance/secretion of animal origin with innumerable therapeutic values. It has been recognized as water of life or "Amrita" (beverages of immotality), the nector of the God. Cow urine has a unique place in Ayurveda and has been described in 'Sushrita Samhita' and 'Ashtanga Sangraha' to be the most effective substance/secretion of animal origin with innumerable therapeutic values. It has been recognized as water of life or "Amrita" (beverages of immotality), the nector of the God[1]. Kamadhenu Ark (distillate) has been shown to have antagonistic effects against the cadmium toxicity in liver of *Mus musculus* and it also work as bioenhancer of Zn[2]. The antitoxic and bioenhancing role of *Kamdhenu Ark*(cow's urine distillate) in fertility rate of male mice affected by cadmium chloride toxicity was studied. The results indicate that Kamdhenu Ark work as an antitoxic against the cadmium chloride toxicity and it can be used as a bioenhancer of Zn[3]. The redistilled cow's urine distillate(RUCD) possesses

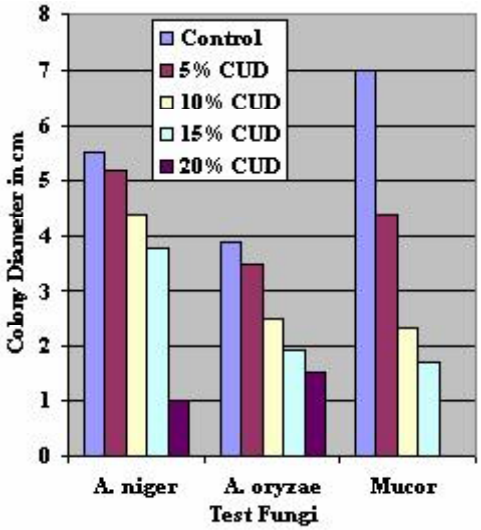

Chart 1: Antifungal activity of different concentrations of Cow urine distillate

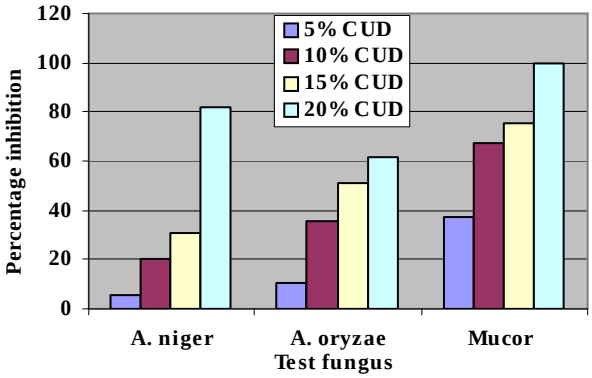

Chart 2: Percent inhibition of test fungi at different concentrations of Cow urine distillate

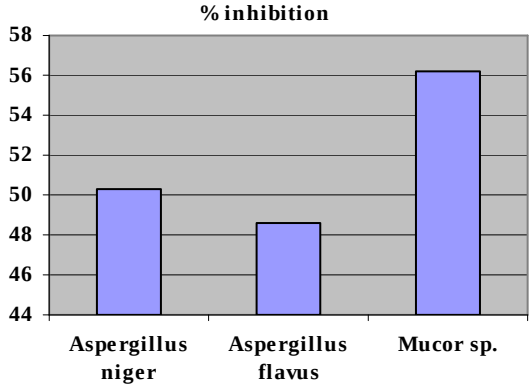

Chart 3: Percentage inhibition of germination of fungal spores by Cow urine distillate as compared to control

strong antigenotoxic and anticlastogenic properties against HPNLs and HLC treated with Cr+6 and MnO2. This property is mainly due to the antioxidants present

in RCUD[4]. Anti-cancer potential of cow urine therapy has been reflected by several case reports, success stories and practical feed back of patients for the treatment of cancer. Cow urine enhances the immunocompetence and improves general health of an individual; prevent the free radicals formation and act as anti-aging factor; reduces apoptosis in lymphocytes and helps them to survive; and efficiently repairs the damaged DNA, thus is effective for the cancer therapy[1]. It has been shown that cow urine has anti-*Leishmania donovoni* effect *in vitro*[5]. Immunomodulatory properties of cow urine on dimethoate induced immunotoxi city in avian lymphocytes using lymphocyte proliferation assay was carried and the results obtained showed that cow urine possess immunopotentiating property[6]. Hepatoprotective activity of Panchagavya Ghrita (PG) against CCl4 induced hepatotoxicity in albino rats was studied. The administration of PG markedly prevented CCl4 induced elevation of levels of serum GPT, GOT, ACP and ALP. Histopathology study of liver exhibited almost normal architecture, as compared to control group[7]. Harmful effects of pesticides and their control through Cowpathy was studied[8]. Screen house experiments were conducted to test the efficacy of cow dung and cow urine separately and in combination in the control of root–knot nematode in tomato. The decrease in the nematode number accompanied by increased plant growth suggests nematicidal potential of urine and Dung[9]. Effect of plant extracts and extracts in combination with cow dung and cow urine on conidial germination of *B.sorokiniana* was studied and the combination trails were found to be superior when compared to activity of only extract[10].

CONCLUSION

The Cow urine distillate was found to possess antifungal activity against fungi tested. The results obtained highlighted an experimental evidence for additional activity of Cow urine distillate in terms of its potential to affect vegetative growth as well as reproductive phase of test fungi. It is proved that Cow urine distillate possess active components found to be inhibitory to the vegetative growth and germination of fungal spores. Cow urine distillate could reduce dissemination of fungal spores as it affected germination of spores. Cow urine distillate could be used as an alternate spraying agent to control plant diseases. Consumption of Cow urine distillate as such or blending it with certain medicinal formulations could elevate host resistance to fungal infections. The test was done *in vitro*. Further studies using animal model could reveal antifungal activity of Cow urine distillate *in vivo*. Experimentally it has been proved that among all sorts of urines, the urine of the Indian cows is most effective. Seeing the potential use of indigenous cow urine in several ailments including even the cancer, the use of Gomutra (cow urine) of indigenous breeds of cattle should be promoted extensively. We conclude that the secretion of cow which is being considered as waste can be used in the control of mycotic infections.

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