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Antifungal effect of cow's urine distillate on *Candida* species

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ABSTRACT

Background: Increase in resistance of *Candida* species, to routinely used antifungal agents has necessitated the quest for new drugs. Few studies have revealed that cow's urine can suppress the growth of pathogenic fungi. However there is no published report on antifungal effects of cow's urine on clinical *Candida* isolates.

Objective: The present study aims at exploring the antifungal potential of cow's urine on clinical isolates of *Candida* species.

Materials and methods: In this *in-vitro* experimental study four standard strains and 37 clinical isolates of *Candida* species were tested for their susceptibility to amphotericin B, fluconazole and voriconazole, by disk diffusion method. Detection of MIC of cow's urine for the *Candida* isolates was done by agar dilution method using 20–50% concentration of cow's urine.

Results: Clinical isolates of *Candida albicans* $n = 22$ (59.5%), *Candida glabrata* $n = 6$ (16.2%), *Candida tropicalis* $n = 3$ (8.1%) and other *Candida* species $n = 6$ were tested for their antifungal susceptibility. Among them, 18.9% were resistant to voriconazole, 24.3% to amphotericin B and 35.1% to fluconazole. Statistically significant association was observed between susceptibility of voriconazole and that of cow's urine ($p = 0.045$). *C. albicans* ATCC14053, *Candida parapsilosis* ATCC22019 and 75.7% of clinical isolates of *Candida* were susceptible to cow's urine.

Conclusion: Cow's urine distillate has concentration dependent inhibitory effect on *Candida* species and is effective on the isolates that are either resistant or sensitive to the routinely used antifungal agents.

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1. Introduction

Continuous emergence of antifungal resistance across the globe has necessitated a quest for new antifungal agents. According to Indian ancient Ayurvedic texts such as “Charak Samhita”, “Ashtanga Sangraha” and “Atharva Veda”, cow's urine has an indelible place in Ayurveda and has been believed to be one of the animal secretions possessing the most therapeutic significances since the ancient time. Cow urine therapy has been practiced by a large number of people for the treatment of various diseases using Panchagavya or Panchakavyam which is made of cow dung, milk, ghee, curd, and urine [1].

Cow Urine Therapy and Research Institute, Indore has shown success in curing ten patients with long term fungal infection of

throat which resisted to ordinary treatment [2]. It has also been used in the treatment of other diseases like hypertension, cancer, diabetes mellitus, ophthalmic disorders, urological syndrome, gynecological disorders, skin diseases, etc. Few studies have also shown the ability of cow's urine in suppressing the growth of pathogenic microorganisms like fungi, bacteria and even helminths [1,3–10]. However a thorough scientific validation is required to establish the efficacy of cow's urine for its worldwide acceptance as an alternative to antifungal agent.

Candidiasis is an opportunistic fungal infection caused by different *Candida* species like *Candida albicans*, *Candida glabrata*, *Candida parapsilosis*, *Candida tropicalis*, and *Candida krusei* which is a normal inhabitant in humans. Invasive candidiasis is a major health care issue in people who are on long term broad spectrum antibiotics treatment, malignancy, immunocompromised state and other predisposing factors [11]. Recent statistics revealed that *Candida* species showed a decrease in susceptibility to azoles such as fluconazole, the most common drug used in the treatment of candidiasis and also to the novel antifungal agent like

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echinocandins. The other alternative such as amphotericin B which are easily available in most of the places but are associated with more adverse effects in comparison with azoles and echinocandins, has now become futile in the treatment of candidiasis caused by some of the *Candida* species, such as *Candida lusitanae* [12]. This emerging antifungal resistance could lead to consequences like administration of more expensive alternative antifungals, prolonged hospitalization, and rise in morbidity and mortality among those high risk individuals.

Hence we aimed at exploring the antifungal potential of cow's urine distillate on *Candida* species isolated from clinical samples which may help to suggest an alternative and cost effective treatment for drug resistant Candidal infections.

2. Materials and methods

This was an *in-vitro* experimental study carried out for a period of four months (01 April 2015 to 30 July 2015) in which distilled cow's urine was tested for its antifungal effect on standard strains and clinical isolates of *Candida* species after obtaining ethical clearance from Institutional Ethics Committee.

2.1. Cow's urine

Distilled cow's urine (known as Arka in Sanskrit) of a special breed (Kapila) of disease free cow, confined mainly to South Karnataka, was obtained from local cow yard at Surabhivana, Kompadavu, Mangalore.

2.2. Strains used in the study

Standard strains like *C. albicans* ATCC 14053 and *C. tropicalis* ATCC 66029 from HiMedia Laboratories Pvt Ltd., *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 from bioMérieux Pvt Ltd. were used to test the anti-fungal effect of cow's urine on these strains and to standardize the amount of urine required for testing clinical *Candida* isolates. Thirty *Candida* species, isolated from different clinical samples were included in the study by following convenient non-random sampling method with 95% confidence level and 90% power with reference to a study conducted by Sathasivam et al. (2010) [1]. Sample size was found to be 30 when calculated using the formula: $n = (Z\alpha^2\sigma^2)/d^2$. All the *Candida* isolates used in the study were identified by standard biochemical reaction [13] and maintained at 4 °C on Sabouraud's Dextrose agar slope.

2.3. Sterility check of cow's urine distillate

Before testing for antifungal effect, sterility check of cow's urine distillate was done by inoculating 1 ml urine distillate into 9 ml of Brain Heart Infusion (BHI) broth. BHI broth was incubated at 37 °C for 4 weeks. At the intervals of 48 h, 4th day 7th day, 14th day, 21st day & 30th day, subcultures were done from the incubated BHI broth onto blood agar and MacConkey's agar and Sabouraud's dextrose agar plates. Inoculated plates were incubated at 37 °C for 48 h and checked for any bacterial or fungal growth [13].

2.4. Detection of MIC of cows urine for standard and clinical strains of *Candida* species

Minimum inhibitory concentration (MIC) of sterile cow's urine preparations on clinically isolated *Candida* species and standard strains of *Candida* species were determined by agar dilution method. Mueller Hinton agar (MHA) containing 0.5 µg/ml methylene blue, 2% glucose and different concentrations of cow's urine distillate (CUD) ranging from 20 to 50% were prepared. Ten

microliters of standard strains of *Candida* species as well as clinical isolates grown in Mueller Hinton broth for 24 h, whose turbidity was adjusted to 0.5 Mac Farland standard (10^6 CFU/ml) was inoculated onto MHA containing different concentration of CUD as well as MHA without CUD. All plates were incubated at 27 °C for 24 h. The highest dilution of the cow's urine that did not show visible growth was taken as MIC. MHA without cow's urine distillate acted as growth control [4,14].

2.5. Susceptibility testing for routinely used antifungal drugs

Anti-fungal susceptibility to routinely used drugs like amphotericin B (100 units), fluconazole (25 µg) and voriconazole (1 µg), was done by disk diffusion method, using Muller Hinton agar supplemented with methylene blue. Results were interpreted as per CLSI guidelines [15,16].

3. Results

3.1. Sterility of cow's urine

Distilled Cow's urine tested were found to be sterile even after 30 days of incubation in BHI broth. These sterility checked urine was used in the study to check their antifungal effect.

3.2. *Candida* species isolated from clinical specimens

A total of 37 *Candida* strains isolated from clinical samples like urine (56.8%), blood (13.5%), sputum (10.8%) and other specimens (18.9%) like ascetic fluid, high vaginal swab, pus, suction tip, central line, endotracheal tube and maxillary sinus were included in the study to know the antifungal effect of cow's urine distillate. These isolates consisted of *C. albicans* (59.5%) followed by *C. glabrata* (16.2%), *C. tropicalis*, *C. krusei*, *C. parapsilosis* and other *Candida* species like *Candida haemulonii*. Age wise and sex wise distribution of *Candida* species among different clinical samples are shown in Tables 1 and 2.

3.3. Effect of cow's urine on standard strains and clinical isolates of *Candida* species

The standard strains of *C. albicans* ATCC 14053, *C. parapsilosis* ATCC 22019, *C. tropicalis* ATCC 66029 and *C. krusei* ATCC 6258 were tested with the routinely used antifungal drugs and cow's urine and the results are shown in Table 3.

Susceptibility to routinely used antifungal agents was found to be interesting. 18.9% of the clinical isolates were resistant to voriconazole (1 µg), 24.3% of the isolates to amphotericin B (100 units) and 35.1% of the isolates to fluconazole (25 µg) (Table 4). However, 24.3% of clinical *Candida* isolates were not inhibited by cow's urine and these included *C. albicans* ($n = 6$), *C. glabrata* ($n = 2$) and *C. krusei* ($n = 1$) (Table 5).

Among the voriconazole resistant clinical isolates, 4 (57.1%) were found to be resistant to cow's urine, whereas 42.9% of isolates which were resistant to voriconazole were sensitive to cow's urine. However, most of the isolates resistant to amphotericin B (5 out of 9 or 55.6%) and fluconazole (8 out of 13 or 61.5%) were found to be susceptible to cow's urine. Moreover, cow's urine was also found to be effective against those clinical isolates that were sensitive to the routinely used antifungal agents. By using Fisher's Exact Test, there was a statistically significant association between susceptibility to voriconazole and that of cow's urine (p value = 0.045, p value less than 0.05 is significant) (Table 6).

Table 1
Distribution of *Candida* species in different age group and sex.

	<i>Candida</i> species (n = 37)						Total
	<i>Candida albicans</i> (n = 22)	<i>Candida glabrata</i> (n = 6)	<i>Candida krusei</i> (n = 2)	<i>Candida tropicalis</i> (n = 3)	<i>Candida parapsilosis</i> (n = 2)	Others (n = 2)	
Age							
1–20	1 (2.7)	0 (0)	1 (2.7)	0 (0)	1 (2.7)	0 (0)	3 (8.1)
21–40	5 (13.5)	0 (0)	0 (0)	0 (0)	0 (0)	2 (5.4)	7 (18.9)
41–60	6 (16.2)	4 (10.8)	1 (2.7)	1 (2.7)	1 (2.7)	0 (0)	13 (35.1)
Above 60	10 (27.0)	2 (5.4)	0 (0)	2 (5.4)	0 (0)	0 (0)	13 (35.1)
Total	22 (59.5)	6 (16.2)	2 (5.4)	3 (8.1)	2 (5.4)	2 (5.4)	37 (100)
Sex							
Male	11 (29.7)	4 (10.8)	1 (2.7)	3 (8.1)	1 (2.7)	1 (2.7)	21 (56.8)
Female	11 (29.7)	2 (5.4)	1 (2.7)	0 (0)	1 (2.7)	1 (2.7)	16 (43.2)
Total	22 (59.5)	6 (16.2)	2 (5.4)	3 (8.1)	2 (5.4)	2 (5.4)	37 (100)

Table 2
Distribution of *Candida* species in various clinical specimen (n = 37).

Clinical samples	<i>Candida</i> species, n (%)						Total
	<i>Candida albicans</i> (n = 22)	<i>Candida glabrata</i> (n = 6)	<i>Candida krusei</i> (n = 2)	<i>Candida tropicalis</i> (n = 3)	<i>Candida parapsilosis</i> (n = 2)	Other (n = 2)	
Urine (n = 21)	14 (37.8)	5 (13.5)	1 (2.7)	1 (2.7)	0 (0)	0 (0)	21 (56.8)
Blood culture (n = 5)	1 (2.7)	0 (0)	0 (0)	1 (2.7)	2 (5.4)	1 (2.7)	5 (13.5)
Sputum (n = 2)	4 (10.8)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	4 (10.8)
Other ^a (n = 9)	3 (8.1)	1 (2.7)	1 (2.7)	1 (2.7)	0 (0)	1 (2.7)	7 (18.9)
Total	22 (59.5)	6 (16.2)	2 (5.4)	3 (8.1)	2 (5.4)	2 (5.4)	37 (100)

^a Other samples include one isolate each from ascetic fluid, high vaginal swab, pus, suction tip, central line, endotracheal tube and maxillary sinus.

Table 3
Susceptibility of standard *Candida* strains to antifungal drugs and cow's urine.

Antifungal agents tested	<i>Candida</i> species (n = 4)			
	<i>Candida albicans</i> ATCC 14053	<i>Candida tropicalis</i> ATCC 66029	<i>Candida krusei</i> ATCC 6258	<i>Candida parapsilosis</i> ATCC 22019
Voriconazole (1 µg)	S	S-DD	S	S
Amphotericin B (100 units)	S-DD	S-DD	S	S-DD
Fluconazole (25 µg)	S	S	S	S
MIC of cow's urine	25%	No inhibition	No inhibition	35%

As per CLSI (Clinical & Laboratory Standards Institute) guidelines: R = resistant; S-DD = susceptible – dose dependent; S = susceptible.

4. Discussion

To the best of our knowledge literature search has not revealed any published data on antifungal effects of cow's urine on clinical isolates of *Candida* species. Hence experiment was standardized initially to know the effect of different concentrations of cow's urine distillate (20–50%) on ATCC *Candida* standard strains and the same was extrapolated to clinical isolates.

4.1. Antifungal action of cow's urine on standard strain of *Candida* species

C. albicans ATCC 14053 was inhibited completely by 25% concentration of cow's urine. This demonstrates that cow's urine has strong inhibitory effect on the growth of *C. albicans* even at low concentration. Similar findings were reported from other studies done on different standard strains like *C. albicans* MTCC 183 [3], *C. albicans* NCIM 1008 [4] and *C. albicans* NCIM 3471 [9].

The growth of *C. parapsilosis* ATCC 22019 was not detected on Mueller Hinton agar with more than 35% concentration of cow's urine. However an earlier study reported cow's urine to be incapable of inhibiting the growth of *C. parapsilosis* MTCC 1965 [3]. This could be due to difference in the *C. parapsilosis* standard strains used in ours and earlier study.

In a study by Vats and Miglani (2011) cow's urine showed no effect on *C. tropicalis* MTCC 184 and *C. glabrata* MTCC 3019 [3]. In the present study, cow's urine distillate concentrations from 25% to 50% was found to be ineffective in suppressing the growth of *C. tropicalis* ATCC 66029 and *C. krusei* ATCC 6258. To the best of our knowledge, published literature are not available to compare the effect of cow's urine on *C. krusei*.

4.2. Antifungal effect of cow's urine on clinical isolates of *Candida* species

Majority of the clinical *Candida* isolates (75.7%) were susceptible to cow's urine in the concentration of 20–50%. Only 24.3% of clinical isolates which consisted of *C. albicans* (n = 6), *C. glabrata* (n = 2) and *C. krusei* (n = 1) were not inhibited by 20–50% concentration of cow's urine. This indicates that higher concentration of cow's urine is required for the growth inhibition of these isolates or these isolates are more resistant to cow's urine than other strains. It was observed that more number of clinical isolates displayed growth inhibition as the concentration of cow's urine increased in the agar plate. This finding reveals that cow's urine distillate has dose dependent antifungal action on *Candida* species as observed by other investigators [1,4–7].

Table 4
Antifungal susceptibility of clinical isolates of *Candida* species.

Routinely used antifungal drugs		<i>Candida</i> species, n (%)						Total
		<i>Candida albicans</i> (n = 22)	<i>Candida glabrata</i> (n = 6)	<i>Candida krusei</i> (n = 2)	<i>Candida tropicalis</i> (n = 3)	<i>Candida parapsilosis</i> (n = 2)	Other (n = 2)	
Voriconazole (1 µg)	R	2 (5.4)	4 (10.8)	1 (2.7)	0 (0)	0 (0)	0 (0)	7 (18.9)
	S-DD	1 (2.7)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (2.7)
	S	19 (51.4)	2 (5.4)	1 (2.7)	3 (8.1)	2 (5.4)	2 (5.4)	29 (78.4)
Total		22 (59.5)	6 (16.2)	2 (5.4)	3 (8.1)	2 (5.4)	2 (5.4)	37 (100)
Amphotericin B (100 units)	R	3 (8.1)	4 (10.8)	1 (2.7)	0 (0)	0 (0)	1 (2.7)	9 (24.3)
	S-DD	17 (45.9)	2 (5.4)	0 (0)	3 (8.1)	2 (5.4)	1 (2.7)	25 (67.6)
	S	2 (5.4)	0 (0)	1 (2.7)	0 (0)	0 (0)	0 (0)	3 (8.1)
Total		22 (59.5)	6 (16.2)	2 (5.4)	3 (8.1)	2 (5.4)	2 (5.4)	37 (100)
Fluconazole (25 µg)	R	6 (16.2)	6 (16.2)	1 (2.7)	1 (2.7)	0 (0)	1 (2.7)	13 (35.1)
	S-DD	1 (2.7)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (2.7)
	S	15 (40.5)	0 (0)	1 (2.7)	2 (5.4)	2 (5.4)	1 (2.7)	23 (62.2)
Total		22 (59.5)	6 (16.2)	2 (5.4)	3 (8.1)	2 (5.4)	2 (5.4)	37 (100)

As per CLSI (Clinical & Laboratory Standards Institute) guidelines: R = resistant; S-DD = susceptible – dose dependent; S = susceptible.

Table 5
Susceptibility of *Candida* isolates to cow's urine (n = 37).

Concentration of cow's urine	<i>Candida</i> species, n (%)						Total
	<i>Candida albicans</i> (n = 22)	<i>Candida glabrata</i> (n = 6)	<i>Candida krusei</i> (n = 2)	<i>Candida tropicalis</i> (n = 3)	<i>Candida parapsilosis</i> (n = 2)	Other (n = 2)	
20%	0 (0)	0 (0)	1 (2.7)	0 (0)	0 (0)	0 (0)	1 (2.7)
25%	5 (13.5)	0 (0)	1 (2.7)	0 (0)	1 (2.7)	1 (2.7)	8 (21.6)
30%	5 (13.5)	0 (0)	1 (2.7)	0 (0)	1 (2.7)	1 (2.7)	8 (21.6)
35%	5 (13.5)	0 (0)	1 (2.7)	0 (0)	1 (2.7)	1 (2.7)	8 (21.6)
40%	16 (43.2)	4 (10.8)	1 (2.7)	3 (8.1)	2 (5.4)	1 (2.7)	27 (73.0)
45%	16 (43.2)	4 (10.8)	1 (2.7)	3 (8.1)	2 (5.4)	2 (5.4)	28 (75.7)
50%	16 (43.2)	4 (10.8)	1 (2.7)	3 (8.1)	2 (5.4)	2 (5.4)	28 (75.7)
No inhibition	6 (16.2)	2 (5.4)	1 (2.7)	0 (0)	0 (0)	0 (0)	9 (24.3)
Total	22 (59.5)	6 (16.2)	2 (5.4)	3 (8.1)	2 (5.4)	2 (5.4)	37 (100)

Table 6
Comparison of susceptibility of *Candida* species to routinely used antifungal agents and cow's urine (n = 37).

Susceptibility to routinely used antifungal agents		Effect of cow's urine, n (%)		Total	p Value by Fisher's exact test
		No inhibition	Has inhibition		
Voriconazole (1 µg)	Resistant	4 (10.8)	3 (8.1)	7 (18.9)	0.045
	Sensitive ^a	5 (13.5)	25 (67.6)	30 (81.1)	
	Total	9 (24.3)	28 (75.7)	37 (100)	
Amphotericin B (100 units)	Resistant	4 (10.8)	5 (13.5)	9 (24.3)	0.178
	Sensitive ^a	5 (13.5)	23 (62.2)	28 (75.7)	
	Total	9 (24.3)	28 (75.7)	37 (100)	
Fluconazole (25 µg)	Resistant	5 (13.5)	8 (21.6)	13 (35.1)	0.229
	Sensitive ^a	4 (10.8)	20 (54.1)	24 (64.9)	
	Total	9 (24.3)	28 (75.7)	37 (100)	

^a Sensitive to routinely used antifungal agents consist of samples that are either susceptible or susceptible – dose dependent based on CLSI (Clinical & Laboratory Standards Institute) guidelines.

Cows urine distillate was found to be effective in inhibiting the growth of drug resistant clinical isolates of *Candida* species [7 isolates resistant to voriconazole (1 µg), 9 isolates resistant to amphotericin B (100 units) and 13 isolates resistant to fluconazole (25 µg)]. This suggests a possibility of use of CUD as an alternative medicine for the drug resistant candida infections which are on the rise across the world due to overuse or misuse of antifungals or antibiotics. By using Fisher's Exact Test, there was statistically significant association between susceptibility to voriconazole and that of cow's urine (p value = 0.045; p value less than 0.05 is significant). On the other hand, the association of susceptibility to amphotericin B and fluconazole with susceptibility to cow's urine was not

statistically significant with p value of 0.178 and 0.229 respectively (p value more than 0.05 is significant).

Among the clinical *Candida* isolates which were sensitive to commonly used antifungal drugs, majority of them were also susceptible to 20–50% of cow's urine. Thus the cow's urine was proved to be effective not only against antifungal resistant clinical *Candida* isolates but also on susceptible ones. This finding is in concurrence with research findings of Rana and De (2013) [9]. The present study has compared the effect of antifungal drugs and CUD on both clinical and standard strains of *Candida* species, as clinical isolates may be more virulent at times and hence may express higher drug resistance. Hence our study reveals the potential of cow's urine

which may be used as an alternative medicine to the presently used antifungal drugs. As this was an *in vitro* experimental study, similar outcomes may be anticipated when cow's urine is used practically in treating candidiasis. The antifungal property of cow's urine can be explained by the presence of non-volatile active constituents [2,5,6,9] like phenolic acids which are found abundant in the chloroform fraction of cow's urine when subjected to high performance liquid chromatographic (HPLC) analysis [10], the antioxidant property [3,7] and the ability to reduce germination of spore [8]. However, the exact mechanism of action of cow's urine in inhibiting the growth of fungi is still not well known and needs to be explored.

The present study was performed on a small group of *Candida* species isolated from few categories of clinical samples. Hence, large number of clinical samples from different human sites and more species of *Candida* needs to be studied. Moreover distilled urine of single species of Indian cow was studied for its antifungal effect. Whether similar effects are observed with urine of other species of cows from India and abroad needs to be studied.

5. Conclusion

From the study it is evident that CUD has concentration dependent inhibitory effect on *Candida* species. Therefore CUD could be an alternative to the presently used antifungal drugs to combat the existing problem of antifungal resistance. Hence, this study is of clinical and public health importance.

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Conflict of interest

None.

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