

## Antibacterial effect of *Hibiscus sabdariffa* (Roselle) extract in synergism with voriconazole and fluconazole against fluconazole-resistant *Candida albicans* isolates: An *in vitro* study.

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### Abstract

According to reports, *Candida albicans* is believed to be the leading cause of fungal urinary tract infections, otherwise known as Candiduria. Prevention of candiduria using natural products is desirable and should be investigated. The current study reports the antimicrobial effect of *H. sabdariffa* (Roselle) extract, a common herbal drink, in combination with voriconazole or fluconazole against *C. albicans* isolates. Six strains of fluconazole-resistant *C. albicans* isolates were obtained from patients with recurrent candiduria. The interaction of *H. sabdariffa* with voriconazole and fluconazole was investigated using checkerboard tests. When the extract was used in combination with voriconazole, a high degree of synergism was observed and it proved to be an encouraging agent against fluconazole-resistant *C. albicans*, *in vitro*. On the other hand, the extract showed indifferent effect with fluconazole. No antagonistic interaction was however, detected in the tested isolates. Further study is required to determine the mechanism of the synergistic action

**Key words:** Fungal, *Hibiscus sabdariffa*, Candiduria, *Candida albicans*, UTIs

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### Introduction

During the past years, there has been a worldwide increase in incidences of candidal urinary tract infections (UTIs). *C. albicans* is reported to be the leading cause of fungal UTIs, also known as Candiduria [1]. The increase in candidal UTIs is, in part, due to the widespread use of broad-spectrum antibiotics and the increasing numbers of immunocompromised patients [2]. Candiduria has raised fears with the international infectious diseases society because of the limited therapeutic choices. The limited success over the previous decade in the prevention and control of fungal UTIs highlights the difficulty of preventing the infection once it has been established [3]. Fungal UTIs may result in serious complications if treated inadequately [4].

Treatment of Candiduria infections is becoming increasingly difficult due to antifungal drug resistance, especially with fluconazole, which is the most commonly used azole for treating candidal UTIs. Monotherapy with other drugs also encounters the same problem [5, 6]. Hence, either a combination of current antifungal drugs with natural products or the development of new drugs is critical for the future and present therapy of fungal infections [2].

Here lies the need to search for alternate safe and effective new natural antifungal agents that have limited or no side effects. The current study was undertaken to evaluate the *in vitro* synergistic action between *H. sabdariffa* extract alone and in combination with fluconazole and voriconazole against fluconazole-resistant *C. albicans* isolated from recurrent UTIs.

*H. sabdariffa* is a popular herbal drink consumed by people around the world and is used in traditional medicine for the treatment of urinary tract infections and hypertension. The brew, usually known as 'karkade', is made from the calyces of the *H. sabdariffa*. A few studies have been carried out to assess the antifungal activities of this plant against some of common human pathogens [7, 8]. However, no studies have reported the combination effect of this extract with other antifungal agents.

### Materials and Methods

#### Preparation of plant extracts

Air-dried Hibiscus calyces were purchased from the local market. The identity of the calyces was confirmed by comparing the collected specimens with those of a known

identity. The extracts were prepared according to a previously described protocol [9]. Using 1L of 80% aqueous methanol (BDH, UK), the phenolics were extracted from 100g of the grinded calyces. The mixture was then sonicated for 20 minutes and then filtered through Whatman no.2 filter paper. The solvents were then evaporated using a rotary evaporator at 40°C under reduced pressure and the extracts were stored in tightly, sealed glass vials and considered to be *H. sabdariffa* extract, which was then used for the antibacterial assays.

#### **Fungal strains and growth conditions**

Six strains of fluconazole resistant *C. albicans*, CA1-CA6 were used in this study. All were obtained from patients with recurrent candiduria infection. Fungal identities were confirmed using standard microbiologic procedures at the Ohad Hospital, Almadinah, Saudi Arabia.

#### **Determination of antimicrobial activity**

Antimicrobial activities of fluconazole, voriconazole and the extract were determined according to the approved CLSI standard reference method [10]. MIC plates were incubated at 35°C for 48hrs. MICs were defined as the lowest concentration of the drug inhibiting cell growth

using MIC-0. MIC-0 was defined as the lowest drug combination resulting in optically clear wells.

#### **Checkerboard assays.**

The interaction of *H. sabdariffa* with fluconazole and voriconazole was investigated by checkerboard tests on 96 well plates, performed according to the approved standard M27-A3 [9]. The fractional inhibitory concentration index (FICI) was calculated as the sum of the fractional inhibitory concentrations (FICs) of either drug ( $MIC_{\text{fluconazole} + H. \text{sabdariffa}} / MIC_{\text{fluconazole}} + MIC_{H. \text{sabdariffa} + \text{fluconazole}} / MIC_{H. \text{sabdariffa}}$ ). The data were classified according to the value of FICI:  $FICI \leq 0.5$ -synergism;  $0.5 < FICI < 4.0$  - indifference; and  $FICI \geq 4.0$  – antagonism [11]. Experiments were repeated for a minimum of three times.

## **Results**

#### **Antifungal activities**

Various levels of the extract MIC's were observed against all the isolates. MIC values ranged from 0.5 to 2 mg/ml. All the isolates showed resistance to fluconazole ( $MIC > 16 \mu\text{g/ml}$ ). All the isolates showed susceptibility to voriconazole with MICs values of  $< 0.016 \mu\text{g/ml}$  ( $P < 0.05$ ). The results are summarized in Tables 1 and 2.

**Table 1.** Interaction of voriconazole and *H. sabdariffa* extract against clinical isolates of *C. albicans* resistant to fluconazole by checkerboard microdilution assay.

Strains	Agent	MIC-0 of each agent		FIC	FICI	Outcome
		Alone	Combination			
CA1	voriconazole	0.032	0.016	0.5	1	Indifference
	Extract	2	1	0.5		
CA2	voriconazole	0.016	0.004	0.25	0.375	Synergy
	Extract	1	0.125	0.125		
CA3	voriconazole	0.008	0.002	0.25	0.312	Synergy
	Extract	2	0.125	0.062		
CA4	voriconazole	0.064	0.008	0.125	0.25	Synergy
	Extract	2	0.25	0.125		
CA5	voriconazole	0.008	0.001	0.25	0.25	Synergy
	Extract	0.5	0.064	0.125		
CA6	voriconazole	0.016	0.002	0.125	0.187	Synergy
	Extract	1	0.062	0.062		

The concentration of voriconazole ( $\mu\text{g/ml}$ ).

The concentration of the Extract ( $\text{mg/ml}$ ).

The effect (outcome) was calculated based on the FICI.

CA; *Candida albicans*.

MIC; Minimum inhibitory concentration.

FIC ; Fractional inhibitory concentrations.

FICI ; Fractional inhibitory concentration index.

**Table 2.** Interaction of fluconazole and *H. sabdariffa* extract against clinical isolates of *C. albicans* resistant to fluconazole by checkerboard microdilution assay.

Strains	Agent	MIC-0 of each agent		FIC	FICI	Outcome
		Alone	Combination			
CA1	Fluconazole	>64	>64	1	2	Indifference
	Extract	2	2	1		
CA2	Fluconazole	32	8	0.25	0.375	Synergy
	Extract	1	0.125	0.125		
CA3	Fluconazole	32	8	0.25	0.75	Indifference
	Extract	2	1	0.5		
CA4	Fluconazole	>64	>64	1	2	Indifference
	Extract	2	2	1		
CA5	Fluconazole	>64	>64	1	1.25	Indifference
	Extract	0.5	0.125	0.25		
CA6	Fluconazole	>64	>64	1	1.125	Indifference
	Extract	1	0.25	0.125		

The concentration of fluconazole ( $\mu\text{g/ml}$ ).

The concentration of the Extract ( $\text{mg/ml}$ ).

The effect (outcome) was calculated based on the FICI.

CA; *Candida albicans*.

MIC; Minimum inhibitory concentration.

FIC ; Fractional inhibitory concentrations.

FICI ; Fractional inhibitory concentration index.

Data on the effectiveness of combining fluconazole and voriconazole with the extract against *C. albicans* has not been previously reported. The results from the checkerboard assay indicate that combinations of fluconazole and the extract have an ‘indifference’ effect. In general, the checkerboard results indicate that combinations of fluconazole and *H. sabdariffa* have no synergism except for strain CA2. The results are presented in Table 1.

We observed that when the extract was associated with voriconazole at different concentrations, there was a reduction of the voriconazole MICs. Table 2 shows the results of the FICIs for the strains tested. In all of the six strains, there was a strong synergistic effect demonstrated by the FICI calculation

## Discussion

So far only a few studies have been carried out to assess the antimicrobial activities of *H. sabdariffa* extract against common fungal pathogens [7, 12, 13].

The high potency of *H. sabdariffa* against these strains is the basis for its use in traditional medicine for treating and preventing UTIs. The mechanism of this action is not completely understood but it has been proposed that the extract contains phenolic compounds including flavonoids and cyaniding which have been reported to exhibit antimicrobial activities [14-16].

In the present study we have demonstrated that the extract is an effective antifungal agent showing strong *in vitro*

antifungal activity against fluconazole-resistant *C. albicans*. The interaction between the extract with the antifungal agents showed a high amount of synergistic effect with voriconazole and indifferent effect with fluconazole. No antagonistic interactions, however, were detected in the tested isolates.

Our findings are encouraging in view of the growing treatment failures and antifungal resistance to *Candida* and suggest a way of treating resistant Candiduria infections through a drug combination approach using natural plant extract.

The use of plant extracts and phytochemicals with known antifungal activities is becoming a very common practice worldwide and is of great therapeutic significance. The identification of new plant extracts with antifungal activities would be of an immense practical use as a safe and effective means of preventing infections [17,18].

The antifungal activities of the *H. sabdariffa* extract that were observed provide basic evidence for the potential effects of this plant for preventing recurrent candiduria caused by *C. albicans* in combination with traditional antifungal drugs.

In conclusion, when used in combination with voriconazole, the extract is an encouraging and safe agent against fluconazole-resistant *C. albicans in vitro* but further studies are required to determine the mechanism of the synergistic action. It also highlights a significant approach of

the plants extract as an antifungal agent. Although the antimicrobial effect of *H. sabdariffa* has been shown *in vitro*, further studies with a more robust methodology would be needed in order to determine the clinical efficacy of the extract to prevent recurrent UTIs. Future studies with a larger number of strains and resistant isolates are of immense importance. Our studies will continue in order to explain the mechanism of action and effects of extract.

### Conflict of interest statement

We declare that we have no conflict of interest.

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