

New initiative on developing fungicides: A traditional perspective

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ABSTRACT

Global change in *Candida* spectrum has been observed in India along with the world due to uncontrolled use of antifungals. *C.albicans* is the common agent for muco-cutaneous and invasive candidiasis. Existing antifungals seems to have failed fighting this opportunistic pathogen resulting in high rates of morbidity and mortality rates (around 30% mortality), indicated by CDC's surveillance data in 2012. *Candida* has become drug resistant due to expression of efflux pumps which causes low drug accumulation, alteration in target proteins and membrane sterol composition. *Tamarajal*, i.e., water stored in copper vessels has been proclaimed as health elixir by ancient *Ayurveda*. Bactericidal property of copper vessels used in Indian household has already been established by the western world. In this paper, we report *tamarajal* as cytotoxic agent and pseudo-morphogenic agent for *Candida*. The change in cell structure exposed to *tamarajal* was visualized under Scanning Electron Microscope. Biofilm eradication capacity was also assessed to avoid infection due to medical devices. This will help us find out a possible alternative to combat candidiasis.

Key words: muco-cutaneous, *C.albicans*, fungicides,

INTRODUCTION

Candidiasis is quite prevalent in patients with suppressed immunity, hematologic malignancies, solid organ transplantation, renal failure, autoimmune disorder with prolonged steroid therapy. Topical spreading of *Candida* is promoted by oral thrush, denture related stomatitis, burn wounds, steroid use, diaper dermatitis, vaginal colonization etc. The infection type could be superficial mucosal or more severe systemic one. The different classes of drugs available need to be administered at higher doses hence comes with hidden side effects. It ranges from mild headache to severe nephrotoxicity and even heart failure.

To avoid these consequences, rather than killing the pathogen, scientists are now more focused on inhibiting the growth and its virulence of the same. As pathogenicity depends on metabolic pathways, invasion related processes, transcription factors etc., designing an alternative that targets any of these aspects can avoid establishment of infection in the host.

U.S. EPA has registered copper as the only solid surface material to kill bacteria. Dry copper surfaces inactivate cells in a process called contact-mediated killing, by inducing cytoplasmic membrane damage (Quaranta *et al.*, 2014). Bactericidal action of copper has been reported on pathogens like Methicillin-resistant *Staphylococcus aureus*, Vancomycin-resistant *Enterococcus*, *Acinetobacterbaumannii*, *Escherichia coli* etc (Kulkarni, 2011). Within 2 hours of contact time, copper can kill 99.9% of bacteria (CDA, 2010). Research has strongly claimed that copper based surfaces in hospitals can reduce nosocomial infections (Michels *et al.*, 2015). Antibacterial efficacy for copper and brass vessels used in India since ancient times has been referred healthy, recognized and reestablished by Western world (Khamisi, 2005), hence, copper may likely become a metal of choice to combat candidiasis, with lesser side effects.

Microbes do not generally develop resistance mechanism to metals like copper as observed in case of antifungal drugs. In contrast to microbes, human skin is not sensitive to copper. Prolonged use of copper intrauterine devices (IUDs) by women worldwide has proved the metal safe for human (Kimmerle *et al.*, 1993). However administering metallic copper based antifungals may result in physical and mental disorders (Jaishankar *et al.*, 2014).

Tamarajal is prepared by storing drinking water in copper vessel overnight. Daily practice of drinking this treasured water balances three *Doshas* in our body (Sharma *et al.*, 2004). It also boost our immunity and helps perpetuate good health. As per our knowledge, no health issues have been addressed so far due to drinking *tamarajal* even for a very long time. As this does not lead to metal accumulation; it can be a possible alternative to existing antifungals.

In this paper, cytotoxicity of *tamarajal* and its implication on morphogenesis of *Candidawas* studied by time kill assay and germ tube inhibition test respectively. Biofilms formed on various medical devices are less susceptible to antifungals are an easy way to invade the host. Here, we have assessed the efficacy of *tamarajalon* biofilm that will help eradication of mature biofilms on such devices.

MATERIAL AND METHODS

1. Preparation of *tamarajal*: *Tamarajal* was prepared by storing distilled water in a thoroughly cleaned copper vessel for maximum 24h.
2. Fungal isolate: *Candida* species from UTI of a patient was obtained from a general hospital, Mumbai. The isolate was cultured and maintained on Sabouraud's Dextrose agar, for 48h and then stored at 4°C for further use.
3. Media and chemicals: All media were purchased from HiMedia, Mumbai. Commercially available Fluconazole (Flu) tablet (50mg) was used as standard drug. For germ tube inhibition test, serum was collected from a local pathology laboratory.
4. Time kill assay: 10^4 cells/ml were exposed to Flu (32mg/L, MIC for non-resistant *Candida* sp.) & *tamarajal* for 4h. At every hour interval, cell viability was evaluated by spreading 100µl suspension on Sabouraud's media. Cytotoxicity was calculated using CFU count on plates after 48h of incubation at 37°C. Controls were maintained for all experiments with distilled water (Ernst *et al.*, 2002).
5. Germ tube inhibition test: Active *Candida* cells at 10^5 cells/ml concentration were exposed to the above mentioned concentration of Flu and *tamarajal* for a standardized period of 4h, followed by incubation in serum for germ tube formation. Percentage inhibition of germ tube was calculated for each sample to determine its efficacy. Control as mentioned above was maintained (Acharya, 2015).
6. *Candida* exposed to *tamarajal* was fixed and visualized under SEM. Morphological effects was studied.
7. Biofilm eradication test: 10^7 cells/ml *Candida* cells in RPMI-1640 were allowed to adhere to glass surface at 37°C and 75rpm for 90min. Media was replaced after removing unattached cells (Lal *et al.*, 2010). The

biofilm formed after 24h was then exposed to *tamarajal* for another 24h. The biofilm was then washed, dried and stained with crystal violet. The absorbed stain was eluted using 95% ethanol and absorbance was measured at 595nm using Nanodrop (MULTISKAN GO, Thermo scientific). Percentage eradication of *tamarajal* exposed biofilm was calculated.

RESULTS & DISCUSSION

The copper content in *tamarajal* was found to be approximately 1mg/L (ICP-AES, IIT, Bombay), after 24hrs. Within 3h, CFU count indicated 72% cytotoxicity in Flu followed by 59% cytotoxicity with *tamarajal* (Fig. 1). Slow decrease in CFU count in control is attributed to the lysis of cells due to osmotic pressure exerted by distilled water. Correlation between time of exposure (in hours) and cytotoxicity was calculated and the values up to 4h are presented in Table1. Perfectly negative correlation in the control and partially negative correlation were evident in both flu and *tamarajal*.

Table1: Correlation between time of exposure (in hours) and cytotoxicity

Samples	r; correlation
Control	-1;Perfectly negative
Flu	-0.84;Partially negative
<i>Tamarajal</i>	-0.93;Partially negative

Table2: Percentage of Germ tube in *Candida*

Control	Flu	<i>Tamarajal</i>
89.67±2.08	19.67±1.16	0±0

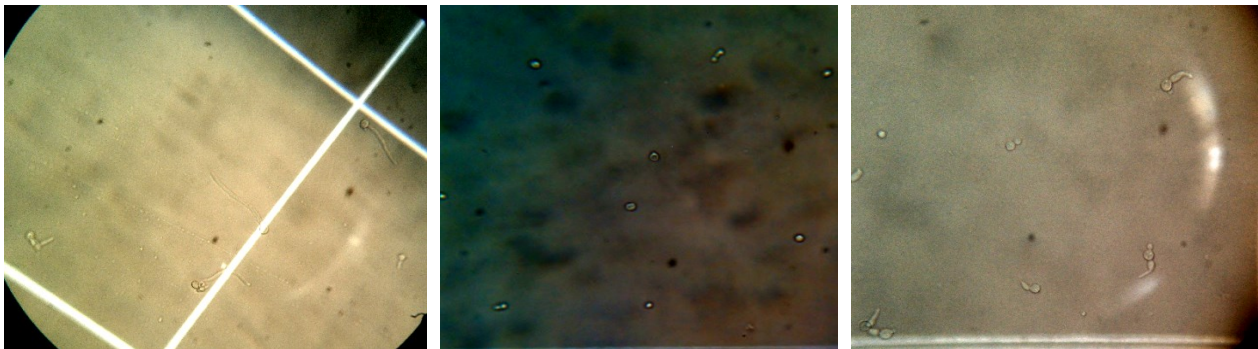
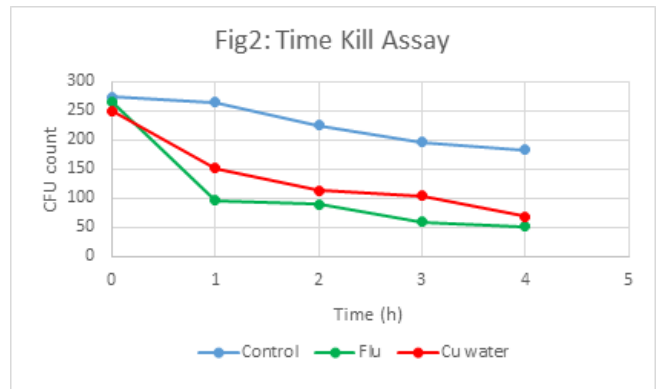


Fig 2: Germ tubes inhibition test: Pre-exposed to A) Control B) Fluconazole C) *tamarajal*

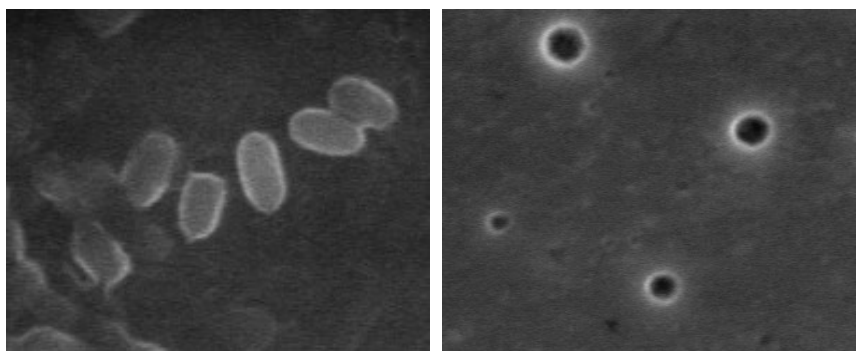


Fig 3: *Candida* under SEM a) control b) *tamarajal*

Inhibition of pathogenicity in *Candida* was effectively and completely observed with *tamarajal*. *Tamarajal* seems to be diverting yeast cells morphogenesis to pseudohyphae rather than true hyphae (Fig 2). The result for germ tube formation are presented in Table 2 with percent mean \pm SD.

Under SEM, the morphological structure of *Candida* cells were detected to be altered when exposed to *tamarajal*. Brightening surface on the *tamarajal* exposed spherical cells confirmed the deposition of copper ions on the cell membrane.

Biofilm eradication capacity of *tamarajalis* well comparable to the standard drug used. *Tamarajal* effectively destroyed biofilm upto 96% which is comparable to Flu that reduced biofilm upto 98%.

DISCUSSIONS

Drug related health issues and increasing resistance towards commonly used drugs by fungal pathogens like *Candida* urge the development of a highly effective yet safe alternative. Copper surfaces have been reported to kill microbial cells by DNA fragmentation, membrane damage, chelating biomolecules, replacing metal ions of some metallo-proteins etc. (Yamanaka *et al.*, 2005). Literature states that copper ion is a non-competitive inhibitor for enzymes, hence permanently damage enzymes. Copper generates hydroxyl radicals which oxidise proteins and helps lipid peroxidation (Hong *et al.*, 2005). Copper ions have also been observed to get internalized causing morpho-structural changes in *E. coli*, causing perturbed structure and cytosolic copper accumulation towards the apical ends (Kambli *et al.*, 2015). The anti-candidal efficacy of copper may be due to a multifaceted action on the cell. Copper ions can be postulated to bind to the negatively charged cell membrane, making it easier for penetration into the cell. It could be due to ROS generation or cytoplasmic membrane damage.

The present study is probably one of the first reports of the anti-candidal effect of *tamarajal*. A healthy practice of drinking *tamarajal* daily regulates thyroid function, slow down aging, helps relieving from pain

and arthritis and stimulate brain. In *tamarajal*, copper gently leaches into the water from the contact surface of the vessel bestowing all its positive properties. As the copper content in *tamarajal* is being well within the normal limits prescribed as 2mg/L by WHO (2003), this proves its safety for application purposes. Detail investigations of *tamarajal* mediated cytotoxicity of *Candida* cells are underway to understand the mode of action.

CONCLUSION

Our results proves that copper in *tamarajal* form, can cause cytotoxicity along with reducing virulence property for pathogenicity. SEM also supports the report by revealing structural alterations of cells, confirming the results. Drug side effects thus could be surpassed using *tamarajal*. It could be used independently or possibly in conjunction with lower doses of existing antifungals to fight pathogens. *In vivo* studies however, must be carried out in future to determine effective dose for the same.

Conflicts of interest: The authors stated that no conflicts of interest.

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