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Targeting fungal menace through copper nanoparticles and *Tamrajal*

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ABSTRACT

Background: WHO reports, an escalation of antibiotic resistance in opportunistic pathogens like *Candida*. *Tamrajal, i.e.,* water stored in copper vessels has been proclaimed as health elixir by ancient Ayurveda. Vis-a-Vis the use of copper contact surfaces and nanoparticles has gained significance for their antimicrobial effects. It thus seems imperative to examine copper nanoparticles and *tamrajal* as promising alternatives to existing antifungals.

Objective: This study not only assessed the influence of *Tamrajal* and copper nanoparticles on the morphological alterations of the *Candida* and its biofilm forming ability, but also on their ability to destroy preformed biofilms.

Materials and methods: Copper oxide nanoparticles as well as *Tamrajal* were evaluated as complementary as well as stand-alone antimicrobial agents. 'Time kill assay' and 'germ tube inhibition test' were performed as end-point analysis for pathogenesis, while biofilm quantification, performed to assess the colonizing capability of *Candida*. Scanning Electron Microscope was used for visualizing the cells, whilst ICP-AES to determine the copper concentration.

Results: 92–100% cytotoxicity to the fluconazole resistant *Candida* species was observed with copper oxide nanoparticles as well as *tamrajal* during 24hr time kill assay. The study also confirmed complete germ tube inhibition by copper in both its forms in addition to the reduction in the biofilm production.

Conclusion: Compared to the classes of antifungals like azoles, echinocandins etc, copper based anticandidal agents highlight a potential way to combat resistant candidiasis. The possibility of accumulation of NP resulting in cytotoxicity puts *tamrajal* as the choice due to its efficacy as well as non-toxicity as per the EPA.

1. Introduction

Tamrajal is prepared by storing drinking water in copper or brass vessel that positively charges the water bestowing all its propitious qualities. Storing water in copper pots finds mention in ancient texts of *Ayurveda* for purification of water [1].

Copper has been registered as the only solid surface material to kill bacteria by the U.S. EPA. Literature also cites the efficacy of metallic copper to kill or inactivate many pathogenic organisms like Methicillin-resistant *Staphylococcus aureus*, Vancomycin-resistant *Enterococcus*, *Acinetobacter baumannii, Escherichia coli* etc. [2,3]. Copper is well known for its biocide mechanisms and it is currently used to fight health-associated (nosocomial) pathogens, foodborne diseases, dust mites loads and fungal and wound infections [4]. Rob Reed claimed that usage of copper alloy like brass vessels especially in developing countries where only basic water purification facilities are possible, combat harmful bacteria [5].

Dry copper surfaces are also known to inactivate yeasts like *Candida albicans* and *Saccharomyces cerevisiae* within minutes in a process called contact-mediated killing, by inducing cytoplasmic membrane damage [6]. Copper coil employed water purification system devised in a laboratory has proved to be highly effective for sanitizing drinking water [7]. Laboratory studies conducted under EPA-approved protocols have proven copper's ability to kill, within 2 h of contact time, more than 99.9% of the disease-causing bacteria [3]. Research has strongly claimed that copper based surfaces in hospitals can reduce nosocomial infections [8]. Its use as bacterial inhibitor in various stages of food processing has been demonstrated against two of the more prevalent bacterial pathogens that cause foodborne diseases, *Salmonella enterica* and *Campylobacter*

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jejuni [9]. Moreover, in contrast to microbes, copper is considered safe for human, as demonstrated by the widespread and prolonged use of copper intrauterine devices (IUDs) by women even in type I diabetic patients [10]. Modified textiles like copper oxide-impregnated apparel products perceived to show biocidal property [11].

Hitherto, effect of *tamrajal* on *Candida* species has not been demonstrated. Although there are few reports available for potential of copper as an antifungal, traditionally used copper water can be an interesting option. As copper is a natural mineral required by our body in trace amount, it could also help in avoiding the adverse effects of existing antifungal drugs.

Rather than killing pathogens, which often require higher dose of drugs, scientists are now focussing on possible drug targets that can inhibit pathogenicity of the organism.

There are more than 300 fungal species responsible for human infection. *Aspergillus, Candida, Cladosporium* are the most common fungal pathogens. Candidiasis is a very common muco-cutaneous infection in India prevalent among the patients with hematic disorders, suppressed immunity, autoimmune disorders, organ transplantation, prolong steroid therapy and with body implants. Nearly 50% mortality is caused by invasive candidiasis. Topical spreading of *Candida* is promoted by nosocomial infections, vaginal colonization, oral thrush etc. Currently available drugs come with their hidden side effects towards the host. In developing countries like India, where antibiotics are used indiscriminately, emergence of drug resistant pathogens is quite common especially in immune-compromised individuals like transplant recipients, HIV/AIDS and cancer patients. Often, switching to more effective antibiotic brings much more complications such as nephrotoxicity, leukopenia, anorexia etc. in patients.

Recently nanotechnology has emerged as the most effective solution in nearly all aspects of life. Incorporation of Ag, Au, Pt, ZnO, TiO_2 nanoparticles in drugs and cosmetics as nanoemulsion, liposome and polymeric nanoparticle formulations has encouraged their use. However, as these have been termed as 'penetration enhancers', attention has been drawn on nanotoxicity upon their prolonged use. Microorganisms do not easily develop resistance power against these metals. Silver, zinc oxide nanoparticles have been reported to possess antifungal activities against fungi like *Penicillium* and *Botrytis* species [12,13]. However, formulation of metal based anti-candidal drugs can also cause metal accumulation in host, which may result in various physical and mental disorders [14]. Since, drinking copper charged water (*Tamrajal*) for a long time does not lead to metal accumulation; it can be a possible alternative to nanoparticles.

Present study aimed to evaluate the efficacy of copper oxide nanoparticles (CuO NPs) and *Tamrajal* on *Candida* species. Microbial cytotoxicity as well as pathogenicity inhibition capacity were analysed for both. Since germ tube is indispensable for tissue invasion in host, inhibition of germ tube formation by a drug is potentially the useful end-point for an antifungal drug assay for *Candida*, than growth inhibition by the same [15]. As *Candida* can easily colonise on biotic and abiotic surfaces by biofilm formation [16], *Tamrajal* in comparison with standard antifungals as well as CuO NPs was investigated for its possible effects on biofilm.

2. Material and methods

2.1. *Preparation of* Tamrajal

Tamrajal was prepared by storing distilled water in a thoroughly cleaned copper vessel for maximum 24 h, as *Ayurveda* recommend storing water overnight before drinking in the morning. Since distilled water is slightly acidic (pH 6.7 ± 0.05) which might enhance copper leaching, we have demonstrated the effect of copper using distilled water to achieve maximum leaching.

2.2. *Fungal isolate*

Clinical isolate of *Candida* species from UTI of a patient was obtained from a general hospital, Mumbai. The isolate was found to be fluconazole resistant and hence cultured on Sabouraud's dextrose agar with fluconazole (32 mg/L), for 48 h and then stored at 4 °C for further use.

2.3. *Media and chemicals*

All media were purchased from HiMedia, Mumbai. Spherical CuO NPs of size 30–50 nm were purchased from Nanolabs, Jamshedpur (India). Commercially available Fluconazole (Flu) tablet (50 mg) was used as standard drug. For germ tube inhibition test, 10 ml of fresh serum was procured from a local pathology laboratory and subsequently used for the study.

2.4. *MIC determination and time kill assay*

MIC of Flu and CuO NPs was determined by following protocol designed under CLSI (M27-A3) [17]. Briefly, dilutions were made using Flu (0.125 μg/ml–64 μg/ml) and CuO nanoparticles (50 μg/ml–500 μg/ml) in RPMI 1640 × 10^4 cells/ml were inoculated in the prepared dilution tubes using Broth Macro-dilution method. The tubes were incubated at 37 °C for 48 h. 80% reduction in growth compared to positive control was determined as MIC for *Candida*. Time point kill assay was performed with required modification in cell density. *Candida* (3 × 10^4 cells/ml) was exposed by suspending active cells in Flu (32 mg/L), CuO NP (300 mg/L) and *tamrajal* (1 mg/L). At every hour interval, cell viability was evaluated by spreading 100 μl suspension on Sabouraud's agar. Cytotoxicity was determined by observing the CFU on plates after 48 h of incubation at 37 °C [18]. Controls were maintained for all experiments with distilled water (since all three samples were present in the same).

2.5. *Germ tube inhibition test*

Determination of pathogenicity of *Candida* species was performed as described by Acharya [19]. 10^5 cells/ml of log phase cells were pre-exposed to the concentrations of Flu, CuO NPs (as determined in the above experiment) and *tamrajal* for a period of 4 h, followed by incubation in serum (procured from a pathological laboratory) at 37 °C to assess its pathogenicity by inducing germ tube formation. Percentage inhibition of germ tube was calculated for each sample to determine its efficacy. Control as mentioned above was maintained.

The experiments were performed in triplicates and the data are expressed as the mean ± standard deviation.

2.6. *Biofilm quantification using crystal violet staining method*

2.6.1. *Biofilm formation ability by pre-exposed cells*

Active *Candida* cells were exposed to the above mentioned concentration of Flu, CuO NPs and *Tamrajal* in borosilicate glass tubes for a defined period of 4 h, followed by wash by phosphate buffered saline (PBS) and inoculation in RPMI-1640 media. Biofilm formation was allowed on pre-sterilized glass tubes [20]. Briefly, 10^7 cells/mL were allowed to adhere to the glass surface by incubation at 37 °C for 90 min at 75 rpm, followed by washing to remove non-adhered cells and addition of fresh RPMI-1640. The tubes were incubated at 37 °C at 75 rpm for another 24hr. After biofilm formation, the media was discarded, biofilm was washed

with PBS and dried at 35 °C for 20 min. Staining of biofilm was performed with 0.4% crystal violet for 20 min, followed by washing with distilled water to remove excess stain. The absorbed stain was eluted out using 95% ethanol and its absorbance was measured at 595 nm using Nanodrop (MULTISKAN GO, Thermo scientific). Absorbance of crystal violet eluted of positive control, i.e. untreated biofilm was considered as 0% inhibition; whereas a glass tube incubated with media under the same conditions and subsequently stained with crystal violet was treated as negative control. Its absorbance was compared with the samples to calculate the percentage inhibition of biofilm using the formula:

$$\%\text{inhibition} = [\text{ABS (control)} - \text{ABS (test)}/\text{ABS (control)}] \times 100$$

2.6.2. Biofilm formation capacity during drug exposure

Candida cells were allowed to form biofilm on glass surface in drug/copper inoculated media as described above. For *tamrajal* effect, media was prepared by replacing deionized water with *tamrajal*. Crystal violet staining and measurement of absorbance of eluted stain was performed to calculate percentage inhibition of biofilm formation.

2.6.3. Biofilm quantitation after drug exposure to preformed film

Biofilms developed as mentioned in 2.6.2 were exposed to the specified concentration of samples after removing media traces by washing thoroughly thrice with distilled water. 24 h treated biofilms were then stained by crystal violet and eluted by 95% ethanol. The eluted stain was read at 595 nm. Percentage eradication of active biofilm by antifungal agent was calculated by comparing it with the controls.

2.7. Scanning electron microscopy of planktonic cells

To reveal cell structural alterations, planktonic cells exposed to all samples were harvested by centrifugation (5000 g/5 min) and fixed using 4% formaldehyde and 1% glutaraldehyde overnight followed by dehydration with a series of ethanol of concentration from 70 to 100%. The cells were visualized using Scanning Electron Microscope (SEM), Quanta 250 at 15 kV.

3. Results

3.1. Copper concentration of water stored in vessel

ICP-AES reveals that *tamrajal* contains approximately 1 mg/L copper, after 24 h of contact time with the vessel [21]. Copper concentration as a function of time of contact with metal surface reaches its optimal concentration in 14 h (0.965 mg/L), as evident from the graph (Fig. 1). Increase in conductivity of water (from distilled water to *Tamrajal*) was observed from 39 μS to 40.7 μS. Reduction in pH of water (from distilled water to *Tamrajal*) from pH 7.3 to pH 7.1 was also observed due to leaching of copper ions.

3.2. Microbial cytotoxicity of Tamrajal

Within 3 h, CFU count indicated 99% cytotoxicity with CuO NPs at 300 mg/L (MIC as determined), compared to 59% cytotoxicity with *tamrajal* at a copper concentration of 1 mg/L as detected by ICP-AES (Fig. 2). The effect of both copper forms was remarkable on this Flu resistant *Candida* at 24 h. Slow decrease in CFU count in control could be attributed to the stressed population of cells due to their suspension in distilled water.

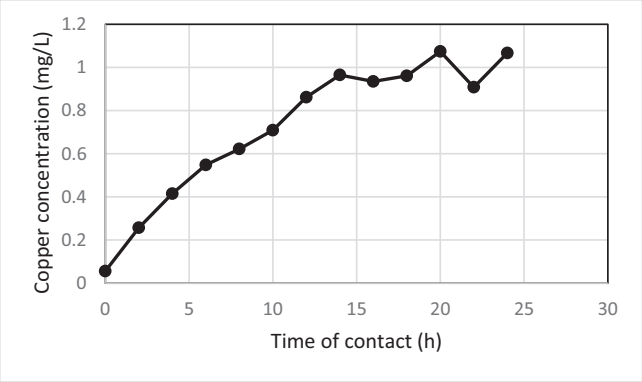

Fig. 1. Copper elution (mg/L) as a function of time for water stored in a copper vessel (for 24 h).

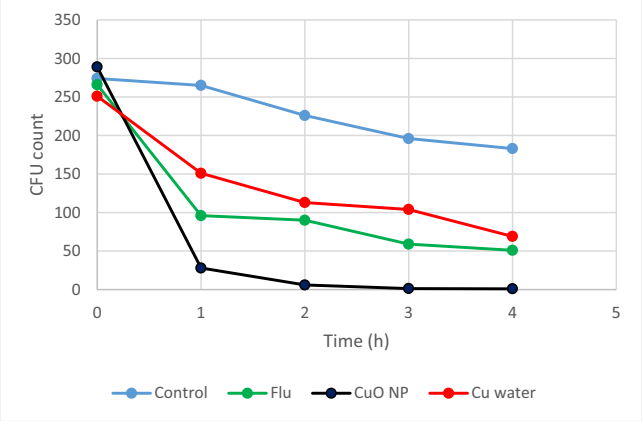

Fig. 2. Time Kill Assay of *Candida* when exposed standard drug (Flu/fluconazole-32 mg/L), CuO NPs (300 mg/L) and *tamrajal* (1 mg/L) in comparison with distilled water as a control.

Correlation between time of exposure (in hours) and cytotoxicity were calculated and the values up to 4 h are presented in Table 1. Perfectly negative correlation in the control and partially negative correlation were evident in all three samples. Using these values, time of exposure required for 100% cytotoxicity of *Candida* were calculated as regression values (in hours) considering x as time(h) dependant on y as CFU count (y as 0 CFU). The values are presented in the Table 1 as $b_{(x \text{ on } y)}$.

3.3. Alterations in pathogenicity

Both CuO NPs and *tamrajal* effectively and completely inhibited true hyphae (germ tube) formation compared to Flu, indicating its efficacy to suppress pathogenicity (Fig. 3). While CuO NP check

Table 1
Correlation between time of exposure of *Candida* to different samples (in hours) and cytotoxic effect induced and their respective regression values.

Samples	r	$b_{(x \text{ on } y)}$
Control	−1; Perfectly negative	10.7 h
Flu	−0.84; Partially negative	3.7 h
CuO NP	−0.76; Partially negative	2.6 h
Cu Water	−0.93; Partially negative	4.9 h

Statistical differences among all four groups were calculated by two way analysis of variance (ANOVA). Differences with $p < 0.05$ (i.e., 0.001375) were considered significant.

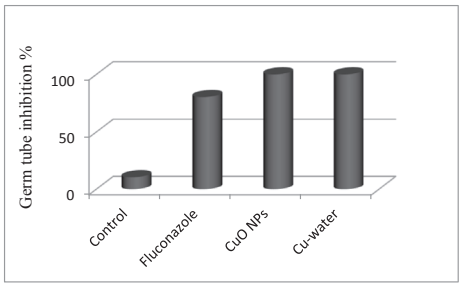

Fig. 3. Germ tube inhibition (%) in *Candida* after exposure to distilled water (control), standard drug (fluconazole- 32 mg/L), CuO NPs (300 mg/L) and *tamrajal* (1 mg/L) for 4 h and induction of germ tube in serum at 37 °C.

pathogenicity by arresting cells in yeast phase, *Tamrajal* worked in a different manner by diverting yeast cells morphogenesis to pseudohyphae rather than true hyphae (Fig. 4). The result for germ tube formation are presented in Table 2 with percent mean ± SD. Graphical representation of germ tube inhibition percentage for all four samples are shown in Fig. 3.

3.4. Effect on biofilm

Graphical representation of percentage inhibition/eradication in biofilm supports the action of *Tamrajal* (Fig. 5). Biofilm eradication capacity of both CuO NPs as well as *tamrajal* is well comparable to the standard drug used. *Tamrajal* was highly effective on prevention of biofilm formation by *Candida* cells when present as a part of the media, compared to Flu and CuO NPs. However, NP-exposed *Candida* cells shows higher reduction in biofilm forming ability than *tamrajal* exposed cells.

3.5. Structural alterations

Morpho-structural changes were evident under SEM showing elongated shapes in *Candida* cells when exposed to CuO-NP at 300 mg/L and Flu at 32 mg/L for 4 h each, while spherical shape was evident in *tamrajal* treated cells, compared to regular oval shape (Fig. 6). Copper deposition on *tamrajal* treated cells can be detected from brightening of the cell surface border.

4. Discussion

Candida has emerged as one of the major problem for nosocomial infections. Its increasing resistance towards commonly used drugs urge the development of a highly effective yet safe alternative.

Table 2
Percentage of Germ tube induced in *Candida* after exposure to distilled water (control), standard drug (fluconazole- 32 mg/L), CuONPs (300 mg/L) and *tamrajal* (1 mg/L) (expressed with S.D.).

Control	Flu	CuO NPs	Cu water
89.67 ± 2.08	19.67 ± 1.16	0 ± 0	0 ± 0

Copper surfaces have been demonstrated to kill vegetative as well as spores of different fungi such as *C. albicans, Aspergillus* species etc. [22]. DNA fragmentation was also evidenced from long term stressed cells. Physical destabilization was also observed due to extended membrane damage upon contact with the metal that results in lysis of the cells [23]. The anti-candidal efficacy of copper may be due to a multifaceted action on the cell. It could be due to ROS generation or cytoplasmic membrane damage. Copper has been proved to induce morpho-structural changes due to internalization of copper ions in *E. coli* leading to its perturbed structure and cytosolic copper accumulation towards the apical ends [24]. Studies have also proposed binding of metal ions to DNA, enzymes and cellular proteins in bacteria, causing cell damage and death [25]. Another study revealed the action of hydroxyl radicals present in the solution for their lethal properties [26]. Copper ions can be postulated to bind to the negatively charged cell membrane, making it easier for penetration into the cell. Copper ions has also been evidenced chelating biomolecules and replace metal ions of some metallo-proteins leading to cell toxicity [27]. Along with microbial eradication, CuO NPs can also lead to acute cellular damage, inflammation, and increase LDH and GGT activity in the host at a concentration as low as 13 mg/m^3. These can also generate reactive oxygen species (ROS) that overwhelm the antioxidant defence of the cell [28]. The toxicity increases with the decrease in particle size due to the ability to consume the hydrogen ions in stomach converting themselves to the more toxic cupric ions [29].

The present study is probably one of the first reports of the anti-candidal effect of *tamrajal*. Drinking *tamrajal* regulates thyroid function, boost immunity, slow down aging, helps relieving from pain and arthritis, synthesize melanin and hemoglobin and stimulate brain.

In *tamrajal*, copper gently leaches into the water from the contact surface of the vessel and lends it all its positive properties. As copper dissolves in water, it becomes ionic (electrolyte) as can be ascertained by its pH measurement. Oligodynamic effect of this water, thus helps in killing microorganisms.

Flu resistant *Candida* was used in the study as fluconazole is the commonly used broad spectrum drug, causing alarming emergence of resistant species. The molecular determination of the isolated *Candida* species is underway. The research has confirmed efficacy of both *tamrajal* and CuO NPs as cytotoxic agents and an effective

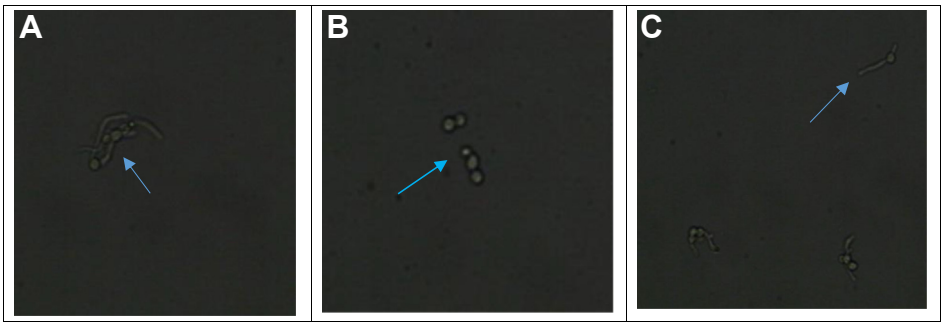

Fig. 4. An untreated *Candida* exhibiting true hyphae (A) as observed under 45X after incubation in serum at 37 °C, (B) *Candida* cells in yeast form as observed post exposure to CuO NPs and (C) Pseudohyphae formation in *Candida* cells exposed to *Tamrajal*.

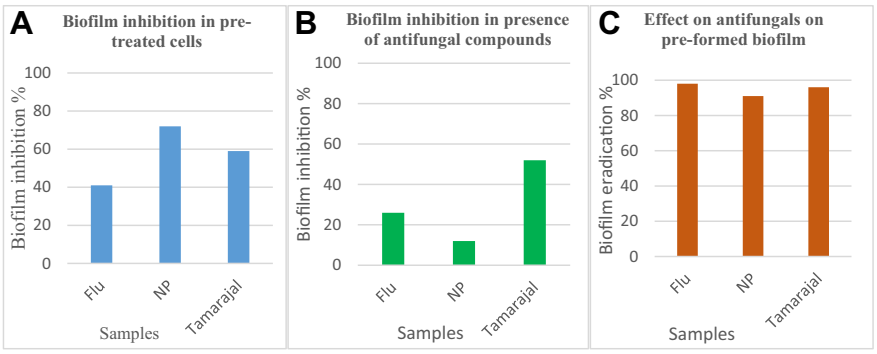

Fig. 5. Inhibition/eradication of *Candida* biofilm as performed and observed under three conditions. A) % Inhibition in biofilm forming of pre-treated candida cells, B) inhibition of biofilm formation due to the presence of the respective antifungals and C) eradication of pre-formed biofilm upon treatment with antifungals (Flu, NP and *tamrajal*).

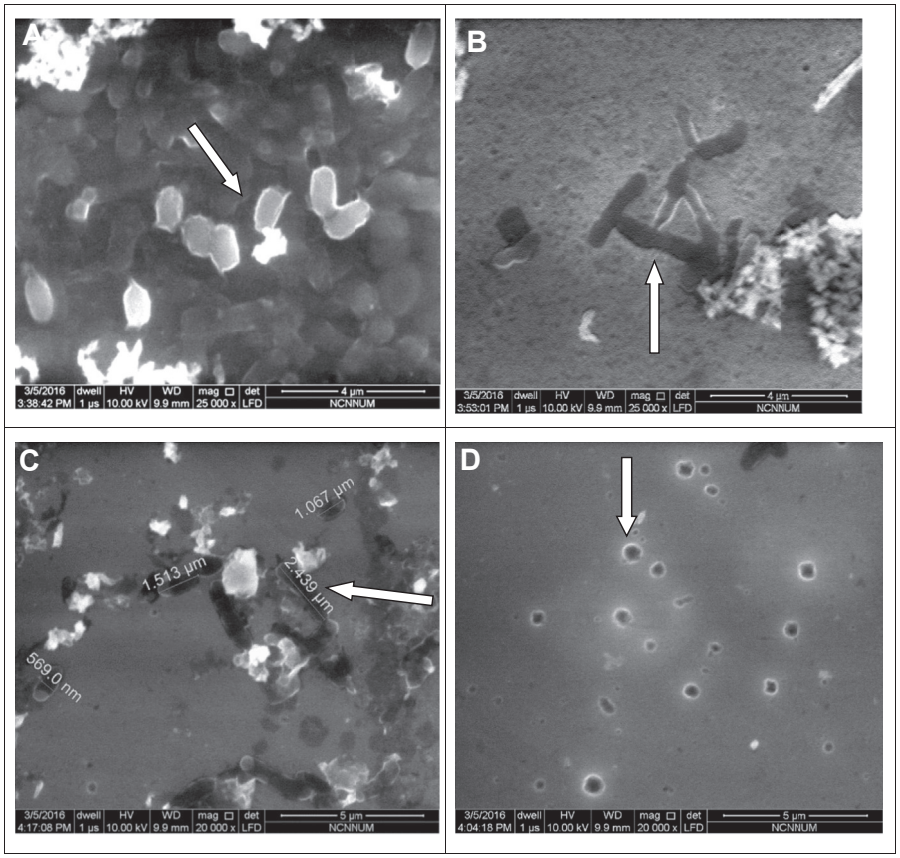

Fig. 6. Scanning electron micrographs of *Candida* kept for 4 h at 37 °C; showing its oval morphology in A) distilled water as control. The cell elongation/alterations observed upon incubation with B) Flu (32 mg/L) and C) CuO NPs (300 mg/L). Spherical shape of the cells with bright cell surface borders as observed when exposed to D) *tamrajal* (1 mg/L).

germ tube inhibitor for the *Candida* species used here. *Tamrajal* at a concentration 300 fold lesser than that of CuO NPs, also proved to be efficient in preventing pathogenesis of this Flu resistant *Candida*. This may be due to the superior action of the element in ionic form that can damage the cells on membrane as well as inside. Detail investigations of *tamrajal* mediated cytotoxicity of *Candida* cells are underway to understand the mode of action.

For *tamrajal*, approximately 5 h exposure time for *Candida* cells is enough for complete clearance of viable ones. Compared to CuO NPs and Flu, though *tamrajal* entail stretched extent of exposure, this could be preferred over both, considering their side effects. As the copper content in *tamrajal* is being well within the normal limits prescribed as 2 mg/L by WHO [30] and 1.3 mg/L by EPA [31], this proves its safety for application purposes.

Biofilm formation ability by pre-exposed cells were studied as this would bring to the notice, the possibility in reduction in colonization of medical devices by *Candida*, upon exposure to copper. Biofilms on such devices can also be reduced if the fungus comes in contact with copper during colonization. Copper could also be possibly applicable to eradicate mature biofilms on devices and within the host. Further studies are in progress to determine the form in which *tamrajal* could be effectively formulated and used.

5. Conclusion

Our results indicated that the NPs as well *tamrajal* not only caused cytotoxicity but also reduced the virulence of *Candida*. The individual effect of the forms of copper on cells seemed to be varied as revealed by microscopy. Elemental or ionic or nanoparticle form of copper thus could be used independently or possibly in conjunction with lower doses of existing antifungals to combat pathogens. *In vivo* studies however, need to be carried out to determine the most effective combination. The study thus paves a new way to the use of different forms of copper in curbing the fungal menace.

Sources of funding

None.

Conflicts of interest

None.

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References

[1] Preethi Sudha VB, Ganesan S, Pazhani GP, Ramamurthy T, Nair GB, Venkatasubramanian P. Storing drinking-water in copper pots kills contaminating diarrhoeagenic bacteria. J Health Popul Nutr 2012;30(1):17–21.
[2] Omprakash, Kulkarni G. Controlling-infections-due-to-hvac-system. Cooling India 2016. Chary Publications.
[3] Antimicrobial Copper FAQs. CDA Publication; 2010. https://www.copper.org/about/pressreleases/2010/pr2010_Sept_15.html.
[4] Borkow G. Using copper to fight microorganisms. Curr Chem Biol 2012;6(2):93–103.
[5] Khamsi R. Brass jugs polish off disease: developing world urged to ditch plastic for traditional pitchers. Nature. ISSN: 1744-7933. EISSN: 1476-4687. Published online 8 April 2005. doi:10.1038/news050404-14.
[6] Quaranta D, Krans T, Espírito Santo C, Elowsky CG, Domaille DW, Chang CJ, et al. Mechanisms of contact-mediated killing of yeast cells on dry metallic copper surfaces. Appl Environ Microbiol 2011;77(2):416–26.
[7] Preethi Sudha VBK, Singh O, Prasad SR, Venkatasubramanian P. Killing of enteric bacteria in drinking water by a copper device for use in the home: laboratory evidence. Trans R Soc Trop Med Hyg 2009;103(8):819–22.
[8] Michels HT, Keevil CW, Salgado CD, Schmidt MG. Copper alloy surfaces kill bacteria and reduce hospital-acquired infections. HERD 2015;9(1):64–79.
[9] Faundez G, Troncoso M, Navarrete P, Figueroa G. Antimicrobial activity of copper surfaces against suspensions of *Salmonella enterica* and *Campylobacter jejuni*. BMC Microbiol 2004;4:19.
[10] Kimmerle R, Weiss R, Berger M, Kurz KH. Effectiveness, safety, and acceptability of a copper intrauterine device (CU Safe 300) in type I diabetic women. Diabetes Care 1993;16(9):1227–30.
[11] Gabbay J, Borkow G, Mishal J, Magen E, Zatcoff R, Shemer-Avni Y. Copper oxide impregnated textiles with potent biocidal activities. J Ind Textil 2006;35(4):323–35.
[12] Kotzybik K, Gräf V, Kugler L, Stoll DA, Greiner R, Geisen R, et al. Influence of different nanomaterials on growth and mycotoxin production of *Penicillium verrucosum*. PLoS One 2016;11(3):e0150855. https://doi.org/10.1371/journal.pone.0150855.
[13] He L, Liu Y, Mustapha A, Lin M. Antifungal activity of zinc oxide nanoparticles against *Botrytis cinerea* and *Penicillium expansum*. Microbiol Res 2011;166(3):207–15.
[14] Jaishankar M, Tseten T, Anbalagan N, Mathew BB, Beeregowda KN. Toxicity, mechanism and health effects of some heavy metals. Interdiscipl Toxicol 2014;7(2):60–72.
[15] Brayman TG, Wilks JW. Sensitive assay for antifungal activity of glucan synthase inhibitors that uses germ tube formation in *Candida albicans* as an end point. Antimicrob Agents Chemother 2003;47(10):3305–10.
[16] Harriott MM, Lilly EA, Rodriguez TE, Fidel PL, Noverr MC. *Candida albicans* forms biofilms on the vaginal mucosa. Microbiology 2010;156(12):3635–44.
[17] CLSI. Reference method for broth dilution antifungal susceptibility testing of yeasts; approved standard-third edition; CLSI document M27-A3. Wayne: Clinical and Laboratory Standards Institute; 2008a. ISBN 1-56238-666-2. Vol. 28 No. 14.
[18] Ernst EJ, Roling EE, Petzold CR, Keele DJ, Klepser ME. In vitro activity of micafungin (FK-463) against Cand*ida sp.*: microdilution, time-kill, and post antifungal-effect studies. Antimicrob Agents Chemother 2002:3846–53.
[19] Acharya T. Germ tube test for Candida: principle, procedure, results and interpretation. Mycology 2015. https://microbeonline.com/germ-tube-test-candida-principle-procedure-results-interpretation/.
[20] Lal P, Sharma D, Pruthi P, Pruthi V. Exo-polysaccharide analysis of biofilm-forming *Candida albicans*. J Appl Microbiol 2010;109:128–36.
[21] Mudiar R, Bhagawat A, Kelkar V. Efficacy of copper charged water against *Candida albicans*. J Food Process Technol 2016;7(5):1–3.
[22] Weaver L, Michels HT, Keevil CW. Potential for preventing spread of fungi in air-conditioning systems constructed using copper instead of aluminium. Lett Appl Microbiol 2010;50:18–23.
[23] Espirito Santo C, Lam EW, Elowsky CG, Quaranta D, Domaille DW, Chang CJ, et al. Bacterial killing by dry metallic copper surfaces. Appl Environ Microbiol 2011;77(3):794–802.
[24] Kambli P, Valavade A, Kothari DC, Kelkar-Mane V. Morphostructural changes induced in *E. coli* exposed to copper ions in water at increasing concentrations. World J Pharmaceut Res 2015;4(10):837–52.
[25] Yamanaka M, Hara K, Kudo J. Bacterial actions of a silver ion solution on *Escherichia coli* studied by energy-filtering transmission electron microscopy and proteomic analysis. Appl Environ Microbiol 2005;71:7589–93.
[26] Espirito Santo C, Taudte N, Nies DH, Grass G. Contribution of copper ion resistance to survival of *Escherichia coli* on metallic copper surfaces. Appl Environ Microbiol 2008;74:977–86.
[27] Chang YN, Zhang M, Xia L, Zhang J, Xing G. The toxic effects and mechanisms of CuO and ZnO nanoparticles. Materials 2012;5:2850–71.
[28] Gosens I, Cassee FR, Zanella M, Manodori L, Brunelli A, Costa AL, et al. Organ burden and pulmonary toxicity of nano-sized coper (II) oxide particles after short-term inhalation exposure. Nanotoxicology 2016;10(8):1084–95.
[29] Meng H, Chen Z, Xing G, Yuan H, Chen C, Zhao F, et al. Ultrahigh reactivity and grave nanotoxicity of copper nanoparticles. J Radioanal Nucl Chem 2007;272(3):595–8.
[30] Copper in drinking-water. Background document for preparation of WHO Guidelines for drinking-water quality. Geneva: World Health Organization; 2003.
[31] National Primary Drinking Water Regulations. https://www.epa.gov/ground-water-and-drinking-water/national-primary-drinking-water-regulations.