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The effect of subinhibitory concentrations of gentian violet on the germ tube formation by *Candida albicans* and its adherence to oral epithelial cells

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ABSTRACT

Objective: This study investigated the effect of subinhibitory concentrations of gentian violet on the germ tube formation by *Candida albicans* and its adherence ability to oral epithelial cells.

Methods: Thirty strains of *C. albicans* isolated from denture wearers, normal healthy individuals and HIV positive patients were used in the study. The antifungal property (Minimum Fungicidal Concentration) of gentian violet was determined at various time intervals using a microdilution technique. The effect of subinhibitory concentrations of gentian violet on the adherence ability (0.000244%) and on germ tube formation ((0.000244%, 0.000122%, 0.000061% and 0.000031%) was determined. In both experiments, water was used as a control. The test results were compared using the Kruskal-Wallis test.

Results: At 60 min a high concentration (0.0078%) of gentian violet was required to completely kill *C. albicans*. Subinhibitory concentrations of gentian violet significantly reduced the adherence ability of *C. albicans* by 57% ($p < 0.01$) and equally inhibited germ tube formation ($p < 0.01$) compared with the controls. The inhibition was concentration dependent, with up to 98% reduction at a concentration of 0.000244%. Germ tube reduction was significantly higher in the isolates from the HIV positive patients than in the isolates from denture wearers.

Conclusion: At high concentrations, gentian violet killed *C. albicans,* whereas at subinhibitory concentrations it reduced its virulence by preventing the adherence ability and germ tube formation. This suggests that the beneficial effects of gentian violet would last beyond the fungicidal concentrations in the treatment of candidiasis.

1. Introduction

Oral candidiasis is an opportunistic fungal infection primarily caused by *Candida albicans,* a unicellular commensal fungus. Its prevalence is therefore high in HIV positive, and cancer and organ transplant patients (Williams & Lewis, 2011). Although it is also found in the oral cavities of healthy individuals, the carrier rate is higher in immunocompromised individuals, particularly HIV positive patients (Patel, Shackleton, & Coogan, 2006). The ability of this opportunistic pathogen to cause infection is due to a number of pathogenic characteristics which facilitate colonisation, penetration and tissue destruction. The first and most essential step in the invasion and colonisation of the epithelial cell is adhesion (Haynes, 2001; Odds, 1994). Tissue invasion is further facilitated by the production of hydrolytic enzymes and the formation of *Candida* hyphae which affects tissue penetration. *Candida albicans* is also capable of adhering to inert materials such as denture acrylic, medical devices and catheters (Calderone & Fonzi, 2001; Cannon & Chaffin, 1999). In addition, novel therapeutic approaches have been suggested where the virulence of *C. albicans* can be targeted (Bein, Schaller, & Korting, 2002; Gauwerky, Borelli, & Korting, 2009; Mehra et al., 2012). Virulence factors such as germ tube formation, adherence ability and the production of tissue damaging hydrolytic enzymes can be the possible targets of such new drugs. Treatment of oral candidiasis includes systemic and topical antifungal agents including mouthwashes. In countries with poor resources many people depend on readily available and cheap remedies such as medicinal plants and chemicals such as gentian violet.

Gentian violet (hexamethyl pararosaniline) is a water soluble triphenymethane dye derived from coal tar. It has been used as an antiseptic for nearly 100 years (Hinton, 1925). In recent years, it has been studied successfully as mono therapy in the treatment of oral candidiasis in HIV infected individuals, especially in developing

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countries (Mukherjee et al., 2017; Wright et al., 2009). Its low cost and ease of application make it a suitable alternative to other topical agents such as nystatin. Nevertheless, given the constant salivary flow in the oral cavity, maintaining gentian violet at the therapeutic concentration is a challenge. In addition, its staining property is a deterrent to compliance. Subinhibitory concentrations of antimicrobial agents such as chlorhexidine, octenidine and pirtenidine are known to inhibit the virulence properties of *C. albicans* (Bobichon & Bouchet, 1987; Ghannoum, Abu Elteen, Stretton, & Whittaker, 1989; Tobgi, Samaranayake, & Macfarlane, 1987). However, the effect of subinhibitory concentrations of gentian violet on the virulence factors including the adherence ability of *C. albicans* has not been studied. Therefore this study investigated the effect of subinhibitory concentrations of gentian violet on the adherence of *C. albicans* to oral epithelial cells, and on its germ tube formation.

2. Materials and methods

2.1. Cultures and inocula

Ten strains of *C. albicans* isolated from the oral cavities of denture wearers, 10 from normal healthy individuals and 10 from HIV positive patients were obtained from the Oral microbiology laboratory. These patients were carriers of *C. albicans*. These cultures had previously been obtained (Owotade, Gulube, Ramla, & Patel, 2016, isolates from denture wearers were from an unpublished study) under ethical clearance (W-CJ-140425-2) and stored at −70 °C. Cultures were grown on Sabouraud dextrose agar at 37 °C for 48 h. For each experiment fresh cultures were used. Medical grade gentian violet BP (0.5%) was purchased from the Parkmed pharmacy, Witbank, South Africa and diluted to obtain different concentrations. For each experiment, fresh inocula was used with an optical density of 0.2 (405 nm) containing approximately 10^5–10^6 organisms per milliliter, prepared from the 48 h cultures.

2.2. Minimum fungicidal concentration (MFC)

An MFC study was performed in order to determine subinhibitory concentrations for the subsequent experiments using a microtitre double dilution technique. Two-fold dilutions of gentian violet (0.25 to 0.000015%) were prepared using Sabouraud broth. One hundred microliters of each of the diluted gentian violet concentrations was added to each of the 16 wells in a 96 well round bottom microtitre plate, into which 100 µl of fresh inocula was added. Plates were incubated aerobically at 37 °C for 24 h. During incubation, each well was sub-cultured on a Sabouraud dextrose agar after 1, 5, 10, 30, 60 and 120 min, and 24 h. The lowest concentration with no growth was recorded as the MFC. For each strain, these experiments were performed three times. Chlorhexidine digluconate (0.2% aqueous solution) was used as a positive control and distilled water as a negative control.

2.3. Adherence assay

Adherence assays were performed using a technique previously described by Patel, Gulube, and Dutton (2009), with modifications. Buccal epithelial cells were collected (by TM) from his own oral cavity as he was a *Candida* non-carrier (ethics waiver no: W-CJ-140425-2). This was done by rubbing gently on the oral buccal mucosa using a sterile cotton wool swab and suspending it in sterile distilled water. Cells were washed by centrifuging at 5000 rpm for 5 min and suspending three times. Washed cells were suspended in 2 ml sterile distilled water.

C. albicans strains were grown onto a Saboraud dextrose broth containing one subinhibitory concentration (0.000244%) of gentian violet for 2 h at 37 °C with constant shaking at 60 rpm. A control containing water instead of gentian violet was also included. After incubation, yeast cells were harvested and centrifuged at 5000 rpm for 5 min, washed three times and re-suspended into 2 ml sterile distilled water. *C. albicans* cells were standardised to 10^7 cells/ml using an haemocytometer. Two millilitres of treated yeast cells and 2 ml of epithelial cells were mixed and incubated at 37 °C for three hours with constant shaking. After incubation, the non-adherent *C. albicans* yeast cells were separated from the epithelial cells using a 20 µm-pore nylon filter paper, washed twice, and the epithelial cells were re-suspended in 2 ml sterile distilled water. Slides were prepared from the suspension and the cells stained with crystal violet for 1 min. One slide per sample was viewed under the microscope. One smear per strain was prepared and viewed twice on different occasions. The mean of the two readings was taken as the final result. The numbers of yeast cells adhering to 100 buccal epithelial cells were counted using light microscopy with a magnification of ×1000. This experiment was performed once for each of the strains of *C. albicans*. The number of adherent yeast cells in the presence of the gentian violet treatment was compared with the control.

2.4. Germ tube formation

The effect on germ tube formation was studied using a technique described by Naicker & Patel in 2013 with modifications. A series of 2 ml horse serum containing 4 different subinhibitory concentrations of gentian violet (0.000244%, 0.000122%, 0.000061% and 0.000031%) were each inoculated with 10 µl of culture inoculum. Horse serum without gentian violet was used as a control. Test tubes were incubated for 3 h at 37 °C. Two hundred microliters of potassium permanganate (5% solution) was added to terminate the metabolic activity and the cultures were left at room temperature for 2 h (Tronchin & Bouchara, 2006). Cells were washed with sterile distilled water. Smears were prepared, heat fixed and the slides were viewed under a light microscope at ×1000 magnification. Fifty cells per smear were randomly selected and the number of germ tubes was recorded. Slides were viewed twice on different occasions. The mean of the two readings was taken as the final result. Cells were considered to have germinated if the germ tube formation was at least twice the length of the cell. These experiments were performed once for each of the strains of *C. albicans* using 4 different concentrations of gentian violet. The numbers of germ tube formations in the presence of subinhibitory concentrations of gentian violet were compared with the control without gentian violet. In addition, the effect on germ tube formation by *C. albicans* isolated from the three study groups was also compared.

2.5. Statistical analysis of data

The effect of gentian violet on the germ tube formation by *C. albicans* and its adherence property was determined by comparing the test results to the control using the Kruskal-Wallis test. The germ tube results were compared between the study groups using a post hoc test. A p value of < 0.05 was considered statistically significant in all the analyses.

3. Results

3.1. Minimum fungicidal concentration (MFC) study

The results of the MFC assays at various time periods are shown in Table 1. The MFC ranged from 0.00003 to 0.0156% of gentian violet solution at 1 min to 24 h. Up to one hour, a high concentration (0.0078%) of gentian violet was required to completely kill *C. albicans* strains isolated from the different study groups. Based on these results 1 subinhibitory concentration (0.000244%) and 4 subinhibitory concentrations (0.000244, 0.000122, 0.000061 and 0.000031%) were selected for the adherence and germ tube assays respectively. Distilled water had no effect on the *C. albicans* cells whereas chlorhexidine digluconate

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