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Evaluation of Antimicrobial Activity of Gold Nanoparticles Against *Candida Albicans* Isolated from Children with Early Childhood Caries

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Abstract

Candida albicans is an opportunistic oral pathogenic fungi and effector in the etiology of early childhood caries (ECC). Antimicrobial resistance (AMR) development has led to a search for potential alternative antimicrobial agents, such as gold nanoparticles (AuNPs). **Objectives**: The aim of this study performed a comparative evaluation of antifungal activity of AuNPs against *C. albicans* isolated from children with ECC and a (0.2% chlorhexidine (CHX)). **Methods**: Oral samples were obtained from 10 children with ECC aged 4-6 years. Samples were cultured on Sabouraud dextrose agar (with 2 g/L chloramphenicol) and incubated at 37°C for 48 h, with *C. albicans* identification based on colony morphology, Gram staining, germ tube formation and analytical profile index (API) *Candida* system biochemical identification. The agar well diffusion method was performed to Test the antifungal activity of AuNPs with concentrations of 0.195 to100 ppm. Positive control was 0.2% CHX solution and negative control was deionized water. **Results**: AuNPs exhibited a concentration-dependent antifungal activity towards *C. albicans*. The maximum one was found out at 100 ppm (23.3 ± 0.06 mm) followed by 50 ppm (20.3 ± 0.06 mm) and 25 ppm (18.7 ± 0.06 mm). With the decrease in AuNP concentration, the inhibition zone gradually declined, which reached 5.7 ± 0.06 mm at concentration of 0.391 ppm. There was no inhibition at 0.195 ppm or deionized water. The inhibition zone of 19.3 ± 0.06 mm was produced by CHX. **Conclusions**: AuNPs exerted strong antifungal activity against oral *C. albicans* isolated from children with ECC in a dose-dependent manner. At higher concentrations, they had an antimicrobial activity exceeding that observed with 0.2% of CHX.

Keywords: AuNPs, CHX, ECC, *C. albicans*, Antimicrobial Activity

Introduction

Candida albicans is a member of the Candidaceae family of polymorphic yeast that is often found as part of the normal microbiota of healthy individuals, colonizing the skin, oral cavity, gastrointestinal tract, and vaginal tract [1]. It is asymptomatic in most individuals (commensal) but will colonize following disruption of the microflora or involving changes in host immunity, and thus, it is considered a pathobiont. The colonization and proliferation of the oral cavity are dependent on many host and environmental factors, such as age, habits, geographical region, socioeconomic status, sex, immunosuppression, and antibiotic exposure [2]. Oral candidiasis is a common localized mycotic disease and can precede the emergence of invasive candidiasis. It can manifest in a number of clinical forms, the most common being pseudomembranous candidiasis. This type causes typical white or creamy patches on the oral mucosa surfaces of the tongue, palate and buccal mucosa, and is called oral thrush [3,4]. In the last 20 years, increasing evidence has suggested that Candida is the fungal genus that can reach high levels of biomass within the oral cavity and would benefit from a low oral pH and conditions that are associated with early childhood caries (ECC) [5]. At the species level, several studies suggest a positive association between the prevalence and abundance of *C. albicans* and occurrence and severity of ECC further suggesting its potential role as an important etiological risk factor [2,6]. Although commonly present in the oral cavity, it was reported previously that *C. albicans* could not colonize to the tooth surfaces effectively on its own, instead, it mainly adheres to oral mucosa and acrylic surfaces, causing mucosal infection. However, others showed that *C. albicans* is capable of adhering to enamel and dentine effectively, and that dental plaque biofilms from children with ECC frequently contain *C. albicans* [6,7].

Emerging evidence has strongly tied the discovery of *C. albicans* in saliva and dental plaque with the development of dental caries, especially in early childhood caries (ECC) pathogenesis. A few cross-sectional studies reported a substantially higher prevalence of oral *C. albicans* in ECC children than caries-free children [2,6,8]. In addition, a systematic review including 15 cross-sectional studies published in 2018 found that children carrying oral *C. albicans* had a greater than five-fold higher odds of developing ECC compared with non-carriers [2]. In agreement with this association, children with oral thrush during their first year of life had more than three-fold higher odds of ECC [6,9]. Furthermore, misuse of chemotherapies and limited availability of vascular-access-protective antifungal treatments remain major public health problems, especially in immuno-compromised populations[10].

The limited availability of antifungal agents has made it increasingly difficult to treat serious *Candida* infections; thus, there is a critical need for new antimicrobial agents with a broader spectrum of antifungal activity and reduced toxicity for human health [9–10]. Nanotechnology have gained much attention and significance in drug design in recent years. Nanoparticles have a small size with a comparably larger surface area, this makes them an attractive platform for diverse biological and industrial applications [11]. Au, Gold NPs are relatively more safe due to its inert and nontoxic character compared to the other inorganic NPs. Moreover, the redox nature of Au has an essential role in decreasing the level of reactive oxygen species (ROS) generation following NP exposure.

Therapeutic uses of gold nanoparticles (AuNPs) or colloidal gold date back over 20 years of use which supports their favorable biocompatibility. Recently conducted studies have also established that AuNPs have significant antifungal activity against various clinical strains of *Candida* and this cuts across indicating their potential as alternative antimicrobial agents [12].

The aim of this study performed a comparative evaluation of antifungal activity of AuNPs against *C. albicans* isolated from children with ECC and a 0.2% chlorhexidine (CHX).

2. Materials and Methods

The study workflow began with the isolation of *C. albicans*, followed by microscopic examination and biochemical testing to confirm its identification. Then, the antifungal activity of the synthesized AuNPs against *C. albicans* was evaluated and compared with 0.2% CHX as a positive control and deionized water as a negative control.

2.1. Isolation of Candida albicans

Samples were obtained from 10 pediatric patients came to the department of pediatric and preventive dentistry in 4-6 years of age with ECC. Children & their parents were told about the oral exam. A written informed consent form that clarifies the purpose of the study and their voluntary well to participate in it was provided for them. Patients were excluded if they were on antifungals, treated with local or systemic fluorides, or had systemic diseases. Diagnosis of caries experience was undertaken by the ICDAS II Sterile cotton swabs were used to take samples from infected areas of the teeth, and the samples were placed into conical tubes containing Sabouraud dextrose medium and conducted to the microbiology laboratory. Subsequently cultivated in Sabouraud dextrose agar medium. Chloramphenicol antibiotic (broad -spectrum antibiotic) [13] was added to the prepared SDA to inhibit the bacterial growth (0.05 of antibiotic to each 1000 ml of medium) and then incubated at 37°C for 48 hrs[14].

2.2. Identification of Candida albicans

2.2.1. Morphological examination

When *C. albicans* was cultured on SDA, colonies were subsequently formed. It is also infrequently possible to distinguish between species of *Candida*. It has Approximately 10% of the collected oral samples have been demonstrated to include more than one species of *Candida*. Recent years have seen an increasing capacity to make discrimination between the different Candida species has taken on increasing significance [15,16].

2.2.2 Microscopical examination

A few inoculums from well-isolated colony was taken using inoculating loop and streaked on clean glass slide. To prepare a uniform suspension, sterile normal saline was added, and the colony material was gently emulsified. The smear was subsequently uniformly spread across the slide and air-dried at room temperature. The smear was then heat-fixed by passing the slide through the hot part of the Bunsen burner flame a few times. Following that, gram staining (performed by standard gram's staining method) was done [17].

2.2.3 Germ tube formation

Suspension of one lope inoculum was obtained by isolation of single colony and emulsified on glass slide with the addition of a drop of normal saline and then spread and kept to air dry at 25C and subjected to fixation through passing of the slide few times over the hot portion of the Bunsen burner flame. This glass slide was stained by Gram's methods [18].

2.2.4. Biochemical Identification:

Identification of fungal isolates and confirmation that it is *C. albicans* was performed with the analytical profile index (API) Candida system (bioMérieux). So, this identification system based on disposable plastic strip, with 10 tubules of very small dimension. Each tubule contains dehydrated substrates. Candida colonies were suspended in 0.85% NaCl with turbidity equivalent to a 3 McFarland standard value which was equal to 106 cell/ml (1-2 isolated and activated colonies) Due to this prepared suspension try to avoid incorporation of air bubbles and immediately covered with mineral oil as some reactions are anaerobic, the API system strip placed at 37°C for 24 hrs. Over the incubation period, a series of metabolic reactions had taken place causing a change on the color of the contents of the tubule.

2.3. Evaluation of antimicrobial effect of Au NPs by using Agar Well Diffusion Test.

The antifungal activity of an AuNP suspension synthesized in a previous study was evaluated on isolated Candida albicans by the agar well diffusion method (19). Overnight cultures of *C. albicans* were suspended in 1 mL of sterile 0.85% NaCl solution and adjusted to 0.5 McFarland turbidity (approximately 1.4×10^{o} CFU/mL). MHA plates were incubated with a standardized inoculum by swabbing the plate surface uniformly using a sterile cotton swab. Then wells of 4 mm in diameter and of uniform depth were made in the agar plate (aseptically), using a sterile stainless-steel cork borer (20). Different concentrations (100, 50, 25, 12.5, 6.25, 3.125, 1.562, 0.781, 0.391 and 0.195) of AuNPs were placed into their respective wells (Figure 1). For the controls, we used 0.2%CHX and deionized water as positive and negative controls, respectively. The experiments were performed in triplicate on three different Petri dishes for each experimental condition (n = 3). The plates were left at room temperature for 10 min following inoculation and then incubated aerobically overnight at 37°C. After incubation, the diameter of the inhibition zones grown around the wells was measured to evaluate the antifungal activity and to determine the minimum inhibitory concentration (MIC) [19].

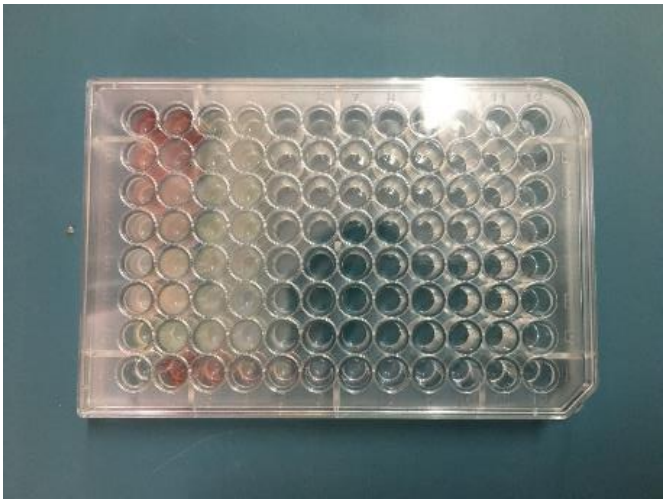

Figure 1: Au NPs at different concentration.

2.4. Statistical Analysis. The data were statistically analyzed and significance at P<0:05 was determined using SPSS software (Statistical for Social Science, Version 22, IBM Corp., USA). The descriptive statistics including mean and standard deviation and least significant difference (LSD) were used to evaluate antimicrobial effect of the Au NPs against the *C. Albicans*.

3. Result

3.1 Morphological Properties of C. albicans

Candida grows as convex, smooth, creamy, and distinct colonies on SDA medium (Figure 2-A). The stained *Candida* with the slide was placed under the light microscope to be identified, which appear as budding yeast cells or gram-positive small oval (Figure 2-B). Germ tubes mean the presence of appendages formed by *C. Albicans* (Figure 2-C). The results of API-*Candida* system reactions were compared with the referring table which provided by the manufacturer and according to it, *C. albicans* were identified (Figure 2-D).

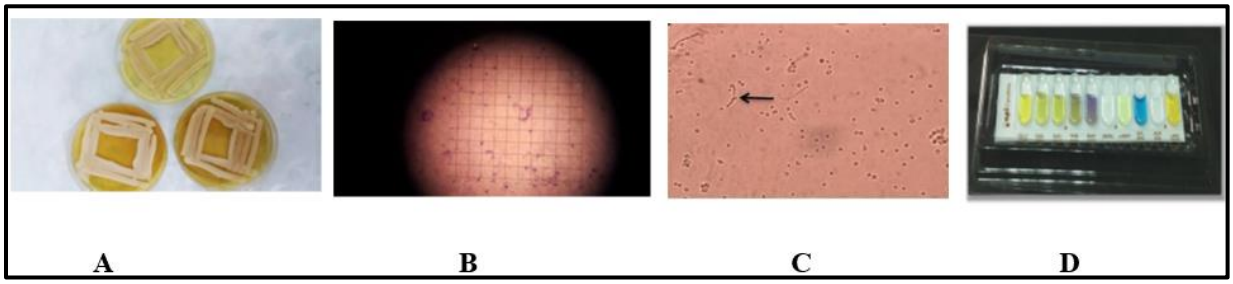

Figure 2: Identification of *Candida albicans:* Colonies of *C. albicans* on SDA(A), *Candida* appears as gram-positive small oval or budding yeast cells (B), Germ tube formation by *C. albicans* (C), API-*Candida* system (D)

3.2. Results of Agar Well Diffusion Method.

Antimicrobial assay revealed concentration-dependent inhibition of *Candida albicans* by gold nanoparticles (AuNPs). The highest inhibition zone was observed at 100 ppm with a clear inhibition zone and inhibitory activity of 23.3 mm as shown in Figure 3. Inhibition zon was decrease when the concentration of AuNPs was reduced gradually, reached to 5.7 mm at 0.391 ppm. This concentration was deemed as the minimum inhibitory concentration (MIC) in the context of the current assay conditions. The antimicrobial efficacy at 0.391 ppm was statistically different from that of chlorhexidine (CHX) ($P < 0.05$). None of AuNPs tested at levels under 0.391 ppm produced inhibition zone, showing no measurable antifungal activity at these lower concentrations. In comparison, the positive control (CHX) generated an inhibition zone with a diameter of 19.3 mm, while the negative control (containing deionized water) ascertained no inhibitory effect against *C. albicans* (Table 1 and Figure 4).

Figure 3: Inhibition zones created by Au NPs against *C. albicans* for concentrations of 100,50,25,12.5,6.25 ppm.

Table 1: Descriptive statistics of antibacterial activity have performed for various concentrations of gold nanoparticles, chlorhexidine and deionized water based on agar diffusion method against *C. albicans*.

Au Concentration	N	Mean (mm)	SD	Minimum (mm)	Maximum (mm)
100	3	23.3	0.06	23.0	24.0
50	3	20.3	0.06	20.0	21.0
25	3	18.7	0.06	18.0	19.0
12.5	3	16.7	0.06	16.0	17.0
6.25	3	14.3	0.06	14.0	15.0
3.125	3	10.7	0.06	10.0	11.0
1.562	3	9.3	0.06	9.0	10.0
0.781	3	8.3	0.06	8.0	9.0
0.391	3	5.7	0.06	5.0	6.0
0.195	3	0.0	0.0	0.0	0.0
CHX (0.2%)	3	19.3	0.06	19.0	20.0
Deionized water	3	0.0	0.0	0.0	0.0

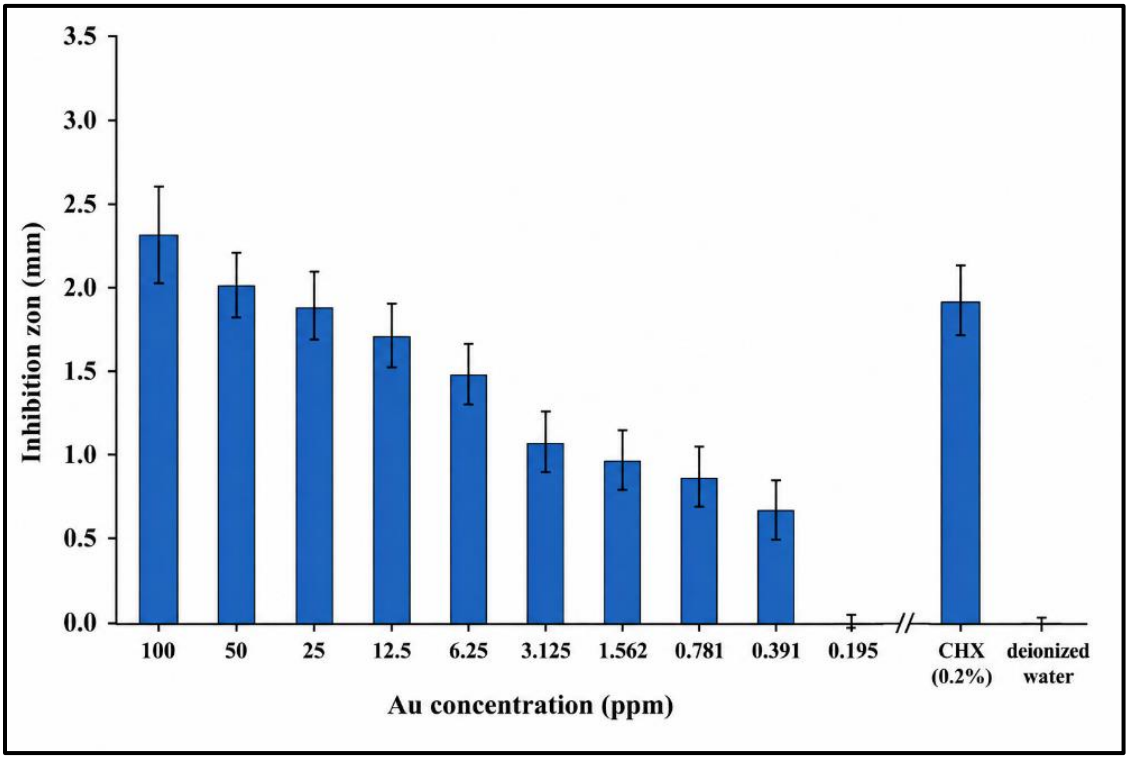

Figure 4: The antimicrobial activity of Au NPs against *C. albicans* P<0:05.

Discussion

In the current study, AuNPs exhibited unambiguous concentration-dependent antifungal activity against *C. albicans* isolated from children with early childhood caries. The well diffusion assay revealed that higher concentrations of AuNPs produced more progressively larger inhibition zones raising from 5.7 mm at 0.391 ppm to 23.3 mm 100 ppm. On the other hand, no inhibition was observed at concentrations lower than 0.391 ppm. The obtained results demonstrate that AuNPs have considerable antifungal activity against *C. albicans*, and the strength of inhibition is a concentration dependent phenomenon.

The greatest inhibition zone was revealed at 100 ppm (23.3 ± 0.06 mm) which was greater than that of 0.2% chlorhexidine (CHX) that exhibited an inhibition zone of 19.3 ± 0.06 mm. The statistical comparison is the result of a post-hoc analysis (following) and should be considered accordingly; however, the numerical differential demonstrates that AuNPs, at concentrations high enough, may exert antifungal effects similar to or exceeding those of CHX. This is especially important because CHX is well established as a potent oral antimicrobial agent. Previous studies demonstrated strong in vitro anti-Candida activity of 0.2% CHX, but the level of inhibition is dependent on formulation, strain, experimental settings and diffusion properties of the tested preparation. Moreover, in vitro studies revealed 0.2% CHX had a zone of inhibition for *C. albicans* of 26 mm and different studies showed strong anti-Candida activity of CHX-containing mouthrinses [21,22].

The current results are consistent with studies indicating the antifungal effect of AuNPs against *C. albicans*. Torabi and Doudi observed the concentration-dependent antifungal effects of AuNPs against 50 *C. albicans* taken from patients with oral candida infection, MIC and minimum fungicidal concentration values of 5.65 and 45.56 µg/mL were reported respectively [23]. Similarly, Emmanuel et al. reported that biologically synthesized AuNPs were investigated against multiple oral microbes, including *C. albicans*, with nontoxic antibacterial activity. Based on their results, they propose that AuNPs may be developed into an alternative antimicrobial agent against microorganisms associated with oral disease [24].

Other studies confirmed direct antifungal effects of AuNPs as well, however, with large discrepancies in effective concentrations [18, 19]. Nidhin et al. reported synthesizing about | 5-nm spherical AuNPs and showed that | 0.5 mM and less were effective against *C. albicans* growth using broth microdilution and spot assays [25]. Dananjaya et al. later found an MIC of 32 µg/mL and a MFC of 64 µg/mL for green-synthesized AuNPs from polysaccharide produced by Spirulina maxima, which had a concentration-dependent antifungal effect against *E. coli* [26]. The discrepancies between these studies and the current results may be attributed to several factors such as the size and morphology, surface chemistry, the synthesis method, stabilizing or reducing agents of the nanoparticles, fungal strains, culture medium and susceptibility-testing methods [17, - 19].

One of the major observations of the current study is that AuNPs strongly inhibited the infectivity from 25 to 100 ppm. As shown, the inhibition zones increased with AuNPs concentration from 18.7 mm at 25 ppm to 23.3 mm at 100 ppm, indicating that increasing the quantity of AuNPs available for interaction with fungal cells effectively promoted growth inhibition. Because the likelihood that a nanoparticle will interact with a cell enlarges with increasing particle concentration, the fact that the effect is dependent on particle concentration is biologically plausible. AuNPs can bind to microbial cells on their cell surface, disrupt cellular behavior and biofilm development. Yu et al. demonstrated that AuNPs can highly bind to *C. albicans* and thus inhibit biofilm formation and invasion, and electrostatic interactions are believed to be an important part of nanoparticle–microorganism interaction [27].

Another mechanism could be intracellular, also mediating the antifungal activity of AuNPs. Previous experimental evidence suggests that AuNPs can induce DNA damage and mitochondrial dysfunction in *C. albicans* and thus can activate an apoptosis-like cell death pathway. Surprisingly, such process was described to take place in the absence of high production of reactive oxygen species, revealing that AuNP antifungal activity may involve more than one mechanistic target on the cell, rather than being a single antimicrobial mechanism. Therefore, the observed progressive inhibition in the present study may indicate that as the concentration of AuNPs increases, several critical cellular processes begin to be disrupted [28].

Furthermore, *C. albicans* targets the oral mucous membrane of children with early childhood caries, and through its ability to interact with cariogenic bacteria and participate in biofilm development, it represents a key organism to be examined with novel antimicrobial strategies. Notably, the susceptibility of *C. albicans* was assessed against agents such as CHX in studies examining specifically children who had early childhood caries and found that *C. albicans* can be isolated from carious children and is susceptible to all agents used in this study [29].

Recent investigations increase the evidence supporting the relevance of AuNPs for oral fungal biofilms. De Alteriis et al. showed that formulations based on AuNPs significantly restricted *C. albicans* biofilm formation and altered biofilm-related gene expression [30]. Correspondingly, AuNPs synthesized by Crinum latifolium observed inhibition zones between 19 - 22 mm against *Candida spp.* and inhibited germ-tube formation and biofilm development; ultrastructural examination revealed alterations of the cell membrane and/or the cell wall of the fungi [31].

Furthermore, AuNPs conjugated with fucoidan were shown to inhibit both single-species and mixed biofilm formed by *C. albicans* and *Streptococcus mutans* in a concentration-dependent manner, thus reflect the polymicrobial nature of dental caries [32].

In the current study, CHX generated a 19.3 mm inhibition area. Thus, this indicates that appropriately chemistries of AuNPs may offer antifungal activity at the same level as a traditional oral antimicrobial agent. However, this finding should not be considered that AuNPs are more clinically than CHX. Nanoparticle aggregation, particle size, surface charge and formulation properties will all affect agar diffusion. Thus, the larger inhibition zone does not imply a more potent fungicide in vivo[33].

Lastly, while the study identifies 0.391 ppm as the lowest concentration tested with measurable inhibition, this concentration should be discussed as the lowest concentration producing an inhibition zone and not an unequivocal MIC. Standard determination of MIC usually involves quantitative broth microdilution procedure [6]. Thus, future studies should solidify the MIC and minimum fungicidal concentration using standardized methods and also characterize nanoparticle size, morphology, surface charge, stability, cytotoxicity to oral tissues, and activity against already-formed *C. albicans* biofilms. These investigations would guide in determining if the promising in vitro antifungal activity seen in the current study can be safely translated to a pediatric dental application.

Conclusion

Gold nanoparticles showed appreciable antifungal activity in a concentration-dependent manner against isolates of *Candida albicans* from children with early childhood caries. Inhibition zone was the highest at 100 ppm, generating 23.3 mm inhibition zone. Higher concentration of AuNPs exhibited higher antifungal activity than 0.2% chlorhexidine. These results support the potential of using AuNPs as a novel antimicrobial agent in pediatric oral health for future clinical trials.

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