Silver Nanoparticle and Plant Molecule Combinations Synergistically Inhibit Drug Resistant Biofilms in Candida albicans

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Abstract

Anti-biofilm activity of silver nanoparticles (SNPs) in combination with four molecules i.e., Geraniol, β-Ionone, Citral and Carvacrol were analysed in a polystyrene microplate based in vitro biofilm model using XTT-metabolic assay. SNPs dosages required for the inhibition of anti-biofilm activity were reduced by the addition of the above molecules. MIC of SNPs against planktonic growth of C. albicans ATCC 90028 was reduced by eight fold in presence of geraniol. This interaction was also synergistic in case of C. albicans clinical isolate GMC 03. Geraniol-SNPs combination was synergistic against developing biofilms in both the strains and reduced the MIC of SNPs by 16 fold in C. albicans ATCC 90028.

Keywords

Candida albicans, Synergy, Silver nanoparticles, Plant molecules, Biofilms

Abbreviations

SNPs: Silver Nanoparticles; MIC: Minimum Inhibitory Concentration; ATCC: American Type Culture Collection; FICI: Fractional Inhibitory Concentration Index; XTT: 2, 3-bis (2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium- 5-carboxanilide

Introduction

Antifungal drugs such as azoles, polyenes, and echinocandins are used to treat infections caused by Candida albicans in humans [1]. The biofilms formed by C. albicans on biotic as well as abiotic surfaces like prosthetic devices are highly resistant to host immune defence's as well antifungal agents. As such there is a need to develop novel drugs to handle, the increasing number of immunocompromised patients to be treated, drug resistance, toxicity and side-effects [2-7]. Innovative strategies such as using synergistic combinations of antifungal drugs, use of virulence factor targeting drugs, drug repositioning are viable options [9, 10]. Use of synergistic drug combinations may be an advantageous strategy for antifungal drug development. It may substantially reduce the dosages of drugs, enhance with increased drug efficacy and lower toxicity. Furthermore, it is a multi target strategy that may prevent development of drug resistance [11]. Molecules
of plant origin are well known for their anti-Candida activity [12-14]. Synergistic combinations of molecules such as thymol, carvacrol, allyl isothiocyanate, berberine, retegolic acid, eugenol, glabridin, baicalin, and berberin with antifungal agents are reported [10, 11, 15].

Silver nanoparticles (SNPs) are studied for their antibacterial as well as for antifungal activities [9, 16, 17]. Although silver nanoparticles exhibit anti-Candida activity, they are also known for their toxicity [18, 19]. It is advisable to invent methods that can reduce the toxicity of silver nanoparticles. Synergistic combination of drugs seems to be a good option for reducing the dosage of silver nanoparticles that may reduce its toxicity and still maintain their antimicrobial potential. In this communication, for the first time, we are reporting the efficacy of combinations of SNPs with geraniol, carvacrol, citral and β-Ionone against planktonic growth and drug resistant biofilm forms of C. albicans.

Materials and Methods

Cultures, culture conditions, media and chemicals

*C. albicans*, ATCC 90028 was purchased from IMTECH (Institute of Microbial Technology), Chandigarh, India. A clinical isolate of *C. albicans* GMC 03 was provided by Government Medical College, Nanded, Maharashtra, India. The culture was grown on YPD agar at 4 °C. YPD (1% Yeast extract, 2% Peptone, and 2% Dextrose) was prepared and used for inoculum preparation. All the ingredients of the medium (1% Mannitol, 1% Nutrient broth, 0.2% K2HPO4), and 2, 3-bis (2-methoxy-4-nitro-sulphonyl) -2H-tetrazolium- 5-carboxanilide (XTT) were obtained from Himedia Chemicals Ltd, Mumbai, India.

Menadione was purchased from Sigma Aldrich Chemicals Ltd, Mumbai, India. A single colony from the YPD agar plates was inoculated in fifty ml of YPD broth and incubated at 30 °C on an orbital shaker, at 120 rpm for 24 h. Cells from the activated culture were harvested by centrifugation for 5 min at 2000 g, washed three times, and resuspended in PBS buffer (10mM phosphate buffer, 2.7 mM potassium chloride and 137 mM sodium chloride, pH 7.4). The molecules used in this work i.e. Citral, Geraniol, Carvacrol and β-Ionone were purchased from Sigma-Aldrich Chemicals Ltd, Mumbai, India and Himedia Chemicals Ltd, Mumbai. Dimethyl sulfoxide (Himedia Chemicals Ltd, Mumbai) was used to dissolve phytochemicals at a final concentration of 1%. SNPs were prepared by using the leaf extract of the plant *Polyalthia longifolia* [9].

Minimum inhibitory concentration for planktonic growth

The effect of silver nanoparticles on the growth of cells of *C. albicans* was studied per the Clinical Laboratory Standards Institute guidelines. Various concentrations of SNPs ranging from 0.75 to 100 μg ml⁻¹ were prepared in Spider medium in non-treated 96-well polystyrene plates (Costar, USA). Wells devoid of test compounds served as controls. 100 μl microtitre of inoculum were added to 100 μl of Spider medium in each well to obtain 1 × 10⁷ cells ml⁻¹. The plates were incubated at 35 °C for 48 hours. Growth was measured by taking the absorbance at 620 nm using a microplate reader (Multiskan EX, Thermo Electron Corp., USA).

Biofilm formation and MIC determination

Biofilms of *C. albicans* were developed on the surface of 96-well polystyrene plates. Cell suspensions of 1 × 10⁷ cells ml⁻¹ were prepared in PBS and 100 μl were inoculated into each well. Plates were incubated at 37 °C for 90 min at 100 rpm to allow attachment of the cells to the solid surface. Non-adhered cells were removed by washing the wells with sterile PBS. Two hundred microlitres of the spider medium were then added to each well and the plates were incubated at 37 °C for 48 h to allow biofilm formation. To analyze its effect on biofilm development, various concentrations of SNPs were prepared in spider medium and added to each well immediately after the adhesion phase and incubated for 48 h at 37 °C. To study the activity against mature *C. albicans* biofilms, SNPs were added to 24 h old mature biofilm and incubated for 48 h. After incubation, the wells were washed to remove any nonattached planktonic cells. Wells were observed for presence or absence of biofilms using an inverted light microscope (Metzer, India). Photographs were taken with a Labomed microphotography system (Labomed, India) at ×200 magnification. Biofilm growth was analyzed and confirmed with the XTT metabolic assay [21].

Biofilm quantitation by XTT assay

To quantify biofilm growth, XTT metabolic assay was performed. The XTT solution was prepared by mixing 1 mg ml⁻¹ of XTT salt in sterile distilled water. Prior to use, menadione solution was prepared in acetone and added to the XTT to a final concentration of 4 μM. The wells containing biofilm were washed with PBS to remove non-adhered cells and incubated with 100 μl of XTT-menadione solution in dark, at 37 °C for 5 h. Color product of the water soluble formazan product was measured at 450 nm using a microplate reader (Multiskan EX, Thermo Electron Corp. USA). Wells without the SNPs were referred as controls. The concentration of SNPs which caused ≥ 50 % lowering in relative metabolic activity was considered the MIC for biofilm formation [21].

Checkerboard assay for drug combination against planktonic and biofilm growth

To study the efficacy of drug combinations against planktonic and biofilm growth in *C. albicans*, checkerboard assay was performed and fractional inhibitory concentration indices (FICI) was analyzed. A two dimensional array of drug combinations were used for the preparation of drug dilutions. The efficacy of drug combinations against planktonic and biofilm growth was performed as discussed in the earlier section and the MIC value were determined. The FICI values were calculated by the formula

\[
2FIC = FIC_A + FIC_B
\]

\[
FIC_A = (MIC of drug A in combination / MIC of drug A alone)
\]

\[
FIC_B = (MIC of drug B in combination / MIC of drug B alone)
\]
FIC\textsubscript{B} = (MIC of drug B in combination / MIC of drug B alone)

When the value of $\Sigma$FICI ≤ 0.5, it was considered as synergism; between 0.5 and 1.0, it was additive; and when $\Sigma$FICI > 4, was considered as antagonism. A $\Sigma$FICI result of >1 but ≤ 4 was treated as indifference [22].

**Results**

**Silver nanoparticles are synergistic with plant molecules against planktonic growth of C. albicans**

Planktonic growth of *C. albicans* was susceptible to the molecules tested. MICs of geraniol, $\beta$-ionone, citral and carvacrol was achieved at 1000 μg/ml, 4000 μg/ml, 500 μg/ml and 250 μg/ml respectively against planktonic growth of *C. albicans* ATCC 90028 (Table 01-4). SNPs showed pronounced inhibitory effect at 1.5 μg/ml against planktonic growth of *C. albicans* ATCC 90028. There was substantial decrease in the dosage of SNPs required for inhibition of planktonic growth of *C. albicans* in presence of the above molecules. For example, MIC of SNPs against planktonic growth of *C. albicans* ATCC 90028 in combination with geraniol was achieved at 0.187 μg/ml i.e. eight times less than that of SNPs alone. Calculated FIC index for the combination was 0.249, suggesting that interaction is synergistic. Interaction was also synergistic in the case of the clinical isolate GMC 03 (Table 1). Addition of 1000 μg/ml of $\beta$-ionone lowered the MIC of SNPs against planktonic growth of *C. albicans* ATCC 90028 from 1.5 to 0.375 μg/ml of SNPs i.e. four fold reduction in MIC of SNPs. FIC index for this interaction was 0.5 indicating that combination is synergistic. This drug combination was also tested against *C. albicans* clinical isolate GMC 03 and calculated FIC was 0.312 showing that the interaction is synergistic (Table 2). In presence of 31.25 μg/ml of citral, MIC of SNPs against planktonic growth of *C. albicans* ATCC 90028 was lowered to 0.375 μg/ml. Combination of SNPs and citral was four times more efficient than that of SNPs alone. Calculated FIC index was 0.3125. This drug combination was synergistic in both the strains of *C. albicans* (Table 3). Carvacrol-SNPs drug combination was not efficient against planktonic growth of *C. albicans* and the calculated FIC index was 1.00 showing that the interaction is indifferent (Table 4).

**Drug combinations of SNP's and selected molecules are additive and synergistic in interaction against developing biofilm of *C. albicans***

Biofilm development was susceptible to SNPs at 50 μg/ml which is 33 fold higher than the MIC for planktonic growth. Developing biofilm was sensitive to the tested molecules and MIC was achieved at 2000 μg/ml, 2000 μg/ml 1000 μg/ml and 500 μg/ml for geraniol, $\beta$-ionone, citral and carvacrol respectively. Geraniol-SNPs combination was highly synergistic against developing biofilms. Addition of 62.5 μg/ml of geraniol reduced the MIC of SNPs against developing biofilm by 16 fold in *C. albicans* ATCC 90028. FIC index for this combination was 0.0937 (Table 1). The MIC of SNPs was decreased from 50 μg/ml to 6.25 μg/ml i.e. eight fold decrease in MIC in presence of 500 μg/ml of $\beta$-ionone in *C.

### Table 01: Comparison of MICs of silver nanoparticles (SNPs) and Geraniol, alone and in combination, against planktonic growth and biofilm of *C. albicans* ATCC 90028 and *C. albicans* GMC 03.

| Growth          | SNPs | Geraniol | SNPs | Geraniol | FICI  | Remarks | SNPs | Geraniol | SNPs | Geraniol | FICI  | Remarks |
|-----------------|------|----------|------|----------|-------|---------|------|----------|------|----------|-------|---------|
| **Planktonic Growth** | 1.5  | 1000     | 0.187| 125      | 0.249 | Synergistic | 3   | 1000     | 0.75 | 3.9      | 0.3   | Synergistic |
| **Biofilm Formation** | 50   | 2000     | 3.125| 62.5     | 0.093 | Synergistic | 50  | 2000     | 6.25 | 500      | 0.375 | Synergistic |
| **Mature Biofilm**  | 100  | 4000     | 6.25 | 1000     | 0.312 | Synergistic | 100 | 4000     | 50  | 125      | 0.531 | Additive |
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Table 02: Comparison of MICs of silver nanoparticles (SNPs) and β-ionone, alone and in combination, against planktonic growth and biofilm of C. albicans ATCC 90028 and C. albicans GMC 03.

| Growth                  | MIC (µg/ml) | Candida albicans ATCC 90028 | Candida albicans GMC 03 |
|-------------------------|-------------|----------------------------|-------------------------|
|                         | Alone       | In Combination             | Alone                   | In Combination             |
| SNPs β-ionone           | 1.5         | 400                        | 0.375                   | 100                       |
| SNPs β-ionone FICI      | 0.5         | Synergistic                | SNPs β-ionone           | 3                         |
| SNPs β-ionone Remarks   | 0.375       | Synergistic                | SNPs β-ionone           | 0.75                      |
| SNPs β-ionone FICI      | 0.375       | Synergistic                | SNPs β-ionone           | 25                        |
| SNPs β-ionone Remarks   | 0.3    | Synergistic                | SNPs β-ionone           | 2000                      |
| SNPs β-ionone FICI      | 0.375       | Synergistic                | SNPs β-ionone           | 0.312                     |
| SNPs β-ionone Remarks   | 0.312       | Synergistic                | SNPs β-ionone           | 250                       |
| SNPs β-ionone FICI      | 0.531       | Additive                   | SNPs β-ionone           | 250                       |

Table 03: Comparison of MICs of silver nanoparticles (SNPs) and Citral, alone and in combination, against planktonic growth and biofilm of C. albicans ATCC 90028 and C. albicans GMC 03.

Table 04: Comparison of MICs of silver nanoparticles (SNPs) and Carvacrol, alone and in combination, against planktonic growth and biofilm of C. albicans ATCC 90028 and C. albicans GMC 03.

out of SNPs-β-ionone caused 8 fold decrease in MIC of SNPs. The calculated FIC index for this drug interaction was 0.375 indicating synergistic interaction. This drug interaction was additive in the clinical isolate, GMC 03 (Table 2). SNPs-citral interaction was additive and FIC index was 0.562 in both the strains of C. albicans (Table 3). Carvacrol combination with SNPs showed indifferent interaction (Table 4).

Discussion

In this study, biosynthesised SNPs were tested against growth and biofilm formation of C. albicans. However, the dosage required to eradicate biofilm was high that may lead to toxicity. Our main thrust was to minimise the dosage of SNPs required for inhibition that can reduce the risk of toxicity. Studies on combinations of silver nanoparticles and molecules of natural origin and their use against C. albicans are lacking. Dosages of SNPs are reduced by the addition of plant molecules and MIC was reduced by several folds (Tables 1–4). This lowered dosage of SNPs may be physiologically relevant as higher dosage may lead to toxicity.

Out of the four molecules tested, geraniol and β-ionone acted synergistically with SNPs against mature and pre-formed biofilm of C. albicans. Addition of geraniol reduced the MIC by sixteen fold while addition of β-ionone reduced the MIC by eight fold against mature biofilms of C. albicans.
(Tables 1 and 2). Use of these drug combinations may be beneficial to treat infections related to biofilms of C. albicans. Physically synthesized SNPs are reported to be cytotoxic against human hepatocellular carcinoma (HepG2) cells above 7.03 ppm of concentration [23]. Silver is eliminated from the human body through the liver and kidneys. Silver is not toxic at low dosages [24]. High concentrations of silver can exert potential toxic side-effects to human cells by preventing DNA and protein synthesis [25]. Studies have shown that silver nanoparticles may be safe at low concentrations [26, 27]. The use of SNPs in combination with plant molecules can be effective to combat Candida infection due its high efficacy and to the fact that dosages of the SNPs can be substantially reduced. There are some reports addressing the synergistic and additive interactions of silver nanoparticles with antifungal agents and antibiotics [9, 13, 28-31]. Silver is well-known for its antimicrobial efficacy even at low concentrations [32]. Currently, silver nanoparticles have gained remarkable attention as antimicrobial agents [33].

Anti-Candida activity of SNPs are reported to be due to the production of ROS, nuclear fragmentation, formation of pores on cell membrane, effect on cell wall [34-38] and their effect on genes involved in signal transduction [39]. The inhibitory activity of plant molecules on C. albicans may be due to membrane damage, inhibition of oxidative phosphorylation and respiration [40]. The mechanism of synergistic interaction of silver nanoparticles with plant molecules is not clear. It seems to be complex and multi-factorial. The mechanism behind the synergistic and additive effect of SNPs-plant molecules may involve attacking of different cellular targets by SNPs and plant molecules in combination. Further in vivo and cytotoxicity studies has to be done in future.

Conclusions

Efficacy of biofabricated SNPs-plant molecule combinations against growth and biofilm formation in C. albicans is presented. Combinations of SNPs –geraniol and SNPs- β-ionone exhibited synergistic inhibitory activity against growth and biofilm forms of C. albicans. These combinations considerably brought down the dosages of SNPs as well as plant molecules and SNPs required for inhibitory activity against biofilms and growth of C. albicans may reduce the risk of toxicities. Further, studies are necessary to test its inhibitory activity in vivo. Detailed toxicity studies are required to be done.

Conflict of interest

No conflict of interest.

Authors contribution

Ashwini Jadhav and Shivkruna Halbandge contributed equally in this publication.

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