

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

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Received: March 30, 2020

Accepted: April 16, 2020

Published: April 17, 2020

**Citation:** Jadhav AK, Halbandge SD, Wakharde AA, Karuppaiy SM. 2020. Silver Nanoparticle and Plant Molecule Combinations Synergistically Inhibit Drug Resistant Biofilms in *Candida albicans*. *NanoWorld J* 6(1): 7-12.

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## Abstract

Anti-biofilm activity of SNPs in combination with four molecules i.e., geraniol,  $\beta$ -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 *Candida 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. Eight fold decrease in MIC was observed in presence of 500  $\mu$ g/ml of  $\beta$ -ionone in *C. albicans* ATCC 90028. MIC of SNPs against mature biofilm forms were brought down by sixteen fold by geraniol. Combination of SNPs- $\beta$ -ionone caused eight fold decrease in the MIC of SNPs against mature biofilm forms in *C. albicans* ATCC 90028.

## Keywords

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

## Introduction

Antifungal drugs such as azoles, polyenes, and echinocandins are used to treat infections caused by *C. 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 immune-compromised 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 very well known for their anti-*Candida* activity [12-14]. Synergistic combinations of molecules such as thymol, carvacrol, allyl isothiocyanate, berberine, retegeric acid, eugenol, glabridin, baicalein, and berberin with antifungal agents were reported [10, 11, 15].

SNPs are studied for their antibacterial as well as antifungal activities [9, 16, 17]. Although SNPs 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 SNPs. Synergistic combination of drugs seems to be a good option for reducing the dosage of SNPs 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  $\beta$ -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 spider medium (1% Mannitol, 1% Nutrient broth, and 0.2%  $K_2HPO_4$ ), and 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 (10 mM 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  $\beta$ -ionone were purchased from Sigma-Aldrich Chemicals Ltd., Mumbai, India and Himedia Chemicals Ltd., Mumbai. Dimethyl sulphoxide (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].

### MIC for planktonic growth

The effect of SNPs on the growth of cells of *C. albicans* was studied as per the Clinical Laboratory Standards Institute guidelines. Various concentrations of SNPs ranging from 0.75 to 100  $\mu\text{g ml}^{-1}$  were prepared in Spider medium in non-treated 96 well polystyrene plates (Costar, USA). Wells devoid of test compounds served as controls. 100  $\mu\text{l}$  of inoculum was added to 100  $\mu\text{l}$  of Spider medium in each well to obtain  $1 \times 10^3$  cells  $\text{ml}^{-1}$ . The plates were incubated at 35 °C for 48 h. Growth was measured by taking the absorbance at 620 nm using a microplate reader (Multiskan EX, Thermo Electron Corp., USA). The lowest concentration of the SNPs which caused a fifty percentage of reduction in the absorbance compared to the control was considered as the MIC for growth of *C. albicans* [21].

### Biofilm formation and MIC determination

Biofilms of *C. albicans* were developed on the surface of 96-well polystyrene plates. Cell suspensions of  $1 \times 10^7$  cells

$\text{ml}^{-1}$  were prepared in PBS and 100  $\mu\text{l}$  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. 200  $\mu\text{l}$  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  $\times 200$  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  $\text{ml}^{-1}$  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  $\mu\text{M}$ . The wells containing biofilm were washed with PBS to remove non-adhered cells and incubated with 100  $\mu\text{l}$  of XTT-menadione solution in dark, at 37 °C for 5 h. Color product by 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  $\geq 50\%$  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 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

$$\Sigma\text{FIC} = \text{FIC}_A + \text{FIC}_B$$

$\text{FIC}_A = (\text{MIC of drug A in combination} / \text{MIC of drug A alone})$

$\text{FIC}_B = (\text{MIC of drug B in combination} / \text{MIC of drug B alone})$

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

## Results

### SNPs were 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  $\mu\text{g/ml}$ , 4000  $\mu\text{g/ml}$ , 500  $\mu\text{g/ml}$  and 250  $\mu\text{g/ml}$  respectively against planktonic growth of *C. albicans* ATCC 90028 (Tables 1-4). SNPs showed pronounced inhibitory effect at 1.5  $\mu\text{g/ml}$  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  $\mu\text{g/ml}$  i.e., eight times less than that of SNPs alone. Calculated FIC index for the combination was 0.249, suggesting that

growth. Developing biofilm was sensitive to the tested molecules and MIC was achieved at 2000  $\mu\text{g/ml}$ , 2000  $\mu\text{g/ml}$ , 1000  $\mu\text{g/ml}$  and 500  $\mu\text{g/ml}$  for geraniol,  $\beta$ -ionone, citral and carvacrol, respectively. Geraniol-SNPs combination was highly synergistic against developing biofilms. Addition of 62.5  $\mu\text{g/ml}$  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  $\mu\text{g/ml}$  to 6.25  $\mu\text{g/ml}$  i.e., eight fold decrease in MIC in presence of 500  $\mu\text{g/ml}$  of  $\beta$ -ionone in *C. albicans* ATCC 90028. Calculated FIC was 0.375 indicating that the interaction was synergistic. However, this combination of drugs were additive in the clinical isolate of GMC 3 (Table 2). Combination of citral and SNPs was not efficient and interaction was indifferent in *C. albicans* ATCC 90028 while this combination was additive in the case of the clinical isolate GMC 03 (Table 3). SNPs-carvacrol drug combination was not effective against developing biofilm of both the strains of *C.*

Table 1: Comparison of MICs of SNPs and geraniol, alone and in combination, against planktonic growth and biofilm of *C. albicans* ATCC 90028 and *C. albicans* GMC 03.

Growth	MIC ( $\mu\text{g/ml}$ )											
	<i>Candida albicans</i> ATCC 90028						<i>Candida albicans</i> GMC 03					
	Alone		In Combination		FICI	Remarks	Alone		In Combination		FICI	Remarks
	SNPs	Geraniol	SNPs	Geraniol			SNPs	Geraniol	SNPs	Geraniol		
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

interaction was synergistic. Interaction was also synergistic in the case of the isolate GMC 03 (Table 1). Addition of 1000  $\mu\text{g/ml}$  of  $\beta$ -ionone lowered the MIC of SNPs against planktonic growth of *C. albicans* ATCC 90028 from 1.5 to 0.375  $\mu\text{g/ml}$  of SNPs i.e., four fold reduction in MIC of SNPs. FIC index for this interaction was 0.5 indicating that combination was synergistic. This drug combination was also tested against *C. albicans* clinical isolate GMC 03 and calculated FIC was 0.312 showing that the interaction was synergistic (Table 2). In presence of 31.25  $\mu\text{g/ml}$  of citral, MIC of SNPs against planktonic growth of *C. albicans* ATCC 90028 was lowered to 0.375  $\mu\text{g/ml}$ . 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 was indifferent (Table 4).

### Drug combinations of SNPs and selected molecules were additive and synergistic in interaction against developing biofilm of *C. albicans*

Biofilm development was susceptible to SNPs at 50  $\mu\text{g/ml}$  which was 33 fold higher than the MIC for planktonic

*albicans*. Interaction of this drug combination was indifferent and calculated FIC was 1.062 (Table 4).

### SNPs and selected molecule combinations act synergistically against mature biofilm forms of *C. albicans*

Mature biofilm forms were susceptible to high dosages of SNPs and MIC was achieved at 100  $\mu\text{g/ml}$  in both the strains of *C. albicans*. MIC against mature biofilm forms were brought down by sixteen fold i.e., from 100  $\mu\text{g/ml}$  to 6.25  $\mu\text{g/ml}$ , in presence of 1000  $\mu\text{g/ml}$  of geraniol. Combination of SNPs with geraniol against mature biofilm of *C. albicans* ATCC 90028 was synergistic and calculated FIC index was 0.3125. However, this drug interaction was additive in case of the clinical isolate GMC 03 (Table 1). Although SNPs alone were effective for the inhibition of biofilm, higher concentrations were required to eradicate the biofilms. 100  $\mu\text{g/ml}$  of SNPs was required for inhibition of 50% of biofilm formation. Combination of SNPs- $\beta$ -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).

**Table 2:** Comparison of MICs of SNPs and  $\beta$ -ionone, alone and in combination, against planktonic growth and biofilm of *C. albicans* ATCC 90028 and *C. albicans* GMC 03.

Growth	MIC ( $\mu\text{g/ml}$ )											
	<i>Candida albicans</i> ATCC 90028						<i>Candida albicans</i> GMC 03					
	Alone		In Combination		FICI	Remarks	Alone		In Combination		FICI	Remarks
	SNPs	$\beta$ -Ionone	SNPs	$\beta$ -Ionone			SNPs	$\beta$ -Ionone	SNPs	$\beta$ -Ionone		
Planktonic Growth	1.5	4000	0.375	1000	0.5	Synergistic	3	4000	0.75	250	0.312	Synergistic
Biofilm Formation	50	2000	6.25	500	0.375	Synergistic	50	8000	25	2000	0.75	Additive
Mature Biofilm	100	8000	12.5	2000	0.375	Synergistic	100	8000	50	250	0.531	Additive

**Table 3:** Comparison of MICs of SNPs and citral, alone and in combination, against planktonic growth and biofilm of *C. albicans* ATCC 90028 and *C. albicans* GMC 03.

Growth	MIC ( $\mu\text{g/ml}$ )											
	<i>Candida albicans</i> ATCC 90028						<i>Candida albicans</i> GMC 03					
	Alone		In Combination		FICI	Remarks	Alone		In Combination		FICI	Remarks
	SNPs	Citral	SNPs	Citral			SNPs	Citral	SNPs	Citral		
Planktonic Growth	1.5	500	0.375	31.25	0.312	Synergistic	3	500	0.375	62.5	0.25	Synergistic
Biofilm Formation	50	1000	50	7.81	1.007	Indifferent	50	1000	25	62.5	0.562	Additive
Mature Biofilm	100	2000	6.25	1000	0.562	Additive	100	2000	6.25	1000	0.562	Additive

**Table 4:** Comparison of MICs of SNPs and carvacrol, alone and in combination, against planktonic growth and biofilm of *C. albicans* ATCC 90028 and *C. albicans* GMC 03.

Growth	MIC ( $\mu\text{g/ml}$ )											
	<i>Candida albicans</i> ATCC 90028						<i>Candida albicans</i> GMC 03					
	Alone		In Combination		FICI	Remarks	Alone		In Combination		FICI	Remarks
	SNPs	Carvacrol	SNPs	Carvacrol			SNPs	Carvacrol	SNPs	Carvacrol		
Planktonic Growth	1.5	250	0.75	125	1.000	Indifferent	3	250	0.187	125	0.562	Additive
Biofilm Formation	50	500	3.125	500	1.0625	Indifferent	50	500	50	7.81	1.015	Indifferent
Mature Biofilm	100	1000	6.25	1000	1.0625	Indifferent	100	1000	100	7.81	1.007	Indifferent

## Discussion

In this study, biosynthesised SNPs were tested against growth and biofilm formation of *C. albicans*. However, the dosage required to irradiate 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 SNPs and molecules of natural origin and their use against *C. albicans* are lacking. Dosages of SNPs were 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  $\beta$ -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  $\beta$ -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 were 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 SNPs 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 SNPs with antifungal agents and antibiotics [9, 13, 28–31]. Silver is well-known for its antimicrobial efficacy even at low concentrations [32]. Currently, SNPs have gained remarkable attention as antimicrobial agents [33].

Anti-*Candida* activity of SNPs were 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 SNPs 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.

## Conclusion

Efficacy of biofabricated SNPs-plant molecule combinations against growth and biofilm formation in *C. albicans* was presented. Combinations of SNPs-geraniol and SNPs- $\beta$ -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.

## 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

## Acknowledgments

SMK and SDH are thankful to UGC-Innovative program, New Delhi for providing financial assistance to SMK.

## Funding

This work was supported by the University Grant Commission –XII plan Innovative Programme, New Delhi, India.

## Conflict of interest

No conflict of interest.

## Authors contribution

Ashwini Jadhav and Shivkrupa Halbandge contributed equally in this publication.

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