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Original Research Paper

In Vitro Evaluation of the Antifungal Activity of Allicin-Loaded β-Cyclodextrin Nanosponges

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Abstract

Allicin, a bioactive sulfur-containing compound derived from garlic (*Allium sativum* L.), possesses promising antifungal activity but its pharmaceutical application is limited by chemical instability. The present study aimed to develop allicin-loaded β-cyclodextrin nanosponges and evaluate their in-vitro antifungal activity against *Candida albicans*. Allicin-loaded nanosponges were prepared by the emulsion solvent diffusion technique using ethyl cellulose, polyvinyl alcohol, and Pluronic F68. The optimized formulation, PF7, containing 24.32 mg allicin, 500 mg ethyl cellulose, 900 mg polyvinyl alcohol, and 200 mg Pluronic F68, was evaluated by agar well diffusion, minimum inhibitory concentration (MIC), and time-kill assays. PF7 exhibited a zone of inhibition of 21.8 ± 0.8 mm compared with 14.2 ± 0.6 mm for free allicin and 24.6 ± 0.5 mm for the standard antifungal. The MIC of PF7 was 62.5 µg/mL, representing a two-fold reduction compared with free allicin (125 µg/mL). In the time-kill study, PF7 produced a 5.20-log10 reduction in viable fungal count after 24 h, compared with 3.40-log10 reduction for free allicin. Thus, nanosponge incorporation enhanced the in-vitro antifungal performance of allicin and represents a promising approach for improving its delivery and efficacy against *C. albicans* under tested conditions.

Keywords: Allicin; β-Cyclodextrin nanosponges; *Candida albicans*; Antifungal activity; MIC; Time-kill assay; Nanocarrier

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Introduction:

Fungal infections are an important cause of morbidity worldwide and range from superficial infections of the skin and mucosal surfaces to life-threatening invasive infections in immunocompromised individuals. Clinically important fungal pathogens include species of *Candida*, *Aspergillus*, *Cryptococcus*, *Trichophyton*, *Microsporum*, and *Epidermophyton*. The management of fungal infections has become increasingly challenging because of the emergence of antifungal resistance, recurrence of infection, adverse effects associated with conventional antifungal agents, and limitations in drug delivery. Azoles, polyenes, echinocandins, and other antifungal drugs remain the major therapeutic options; however, reduced susceptibility and resistance among pathogenic fungi have increased the need for alternative antimicrobial agents. Consequently, naturally derived bioactive compounds are receiving considerable attention as potential sources of novel antifungal agents.Allicin is one of the most important biologically active sulfur-containing compounds associated with garlic (*Allium sativum* L.). It is formed enzymatically from alliin when garlic tissue is disrupted through the action of the enzyme alliinase [1,2]. Allicin contributes substantially to the characteristic biological properties of freshly crushed garlic and has demonstrated antibacterial, antifungal, antiparasitic, antioxidant, and other pharmacological activities [1,2]. Its antimicrobial activity is primarily attributed to its highly reactive sulfur functionality, which allows it to interact with thiol-containing compounds and proteins. Such interactions can modify the function of essential cellular enzymes and interfere with metabolic processes required for microbial survival [2].

The antifungal potential of allicin has been demonstrated against several pathogenic fungi. Yamada and Azuma reported that allicin exhibited in-vitro activity against *Candida*, *Cryptococcus*, *Trichophyton*, *Epidermophyton*, and *Microsporum*, with minimum inhibitory concentrations varying according to the organism and test conditions [3]. These findings established the broad antifungal potential of allicin and provided an important basis for subsequent investigations. Allicin has also demonstrated activity against *Candida albicans*, an opportunistic fungal pathogen responsible for superficial, mucosal, and invasive candidiasis [4]. Furthermore, experimental studies have indicated that allicin may interfere with fungal growth, cellular metabolism, and biofilm-associated processes, thereby supporting its potential as a naturally derived antifungal compound [4,5].The antifungal action of allicin is closely associated with its ability to react with sulfhydryl groups present in cellular proteins. Allicin can undergo thiol-disulfide exchange reactions with cysteine residues, resulting in modification of protein function and disruption of essential metabolic pathways [2]. Important thiol-containing enzymes and proteins involved in cellular redox balance and metabolism may therefore be affected by allicin. Such biochemical interference can disturb cellular homeostasis and contribute to fungal growth inhibition. Studies have further suggested that oxidative stress and cellular damage may participate in the antimicrobial action of allicin [2,5]. However, despite its promising biological activity, the pharmaceutical utilization of allicin is limited by its chemical reactivity and instability.Allicin is a relatively unstable molecule and can undergo transformation into other sulfur-containing compounds depending on environmental and storage conditions [1,2]. Factors such as temperature, oxygen, light, pH, and formulation conditions can influence its stability and consequently its biological availability. Therefore, maintaining the active form of allicin during storage and delivery represents a significant challenge. A suitable carrier system capable of protecting allicin and maintaining its availability may therefore be beneficial for exploiting its antifungal potential.

Cyclodextrin-based nanosponges have emerged as promising nanostructured delivery systems for improving the physicochemical and biopharmaceutical properties of active compounds. Nanosponges are cross-linked, porous polymeric structures containing nanosized cavities capable of incorporating a wide range of molecules [6,7]. β-Cyclodextrin is particularly useful in the preparation of such systems because its cyclic structure contains a relatively hydrophobic internal cavity and hydrophilic outer surface, enabling interactions with different guest molecules [7,8]. Cross-linking of β-cyclodextrin produces a three-dimensional polymeric network with interconnected cavities and a high surface area, providing opportunities for drug or bioactive compound incorporation.

β-Cyclodextrin nanosponges have been investigated as carriers for improving the solubility, stability, protection, and release characteristics of active substances [6-9]. Their porous structure can accommodate both hydrophilic and lipophilic molecules, while the degree and nature of cross-linking can influence their loading capacity and release behavior [7,8]. These characteristics make β-cyclodextrin nanosponges particularly attractive for the delivery of unstable natural compounds. Encapsulation or incorporation of allicin into such a nanosponge system may provide a protective

microenvironment and potentially improve the persistence and availability of the active compound.

In-vitro antifungal assays, including agar or well diffusion techniques, broth dilution, minimum inhibitory concentration (MIC), and minimum fungicidal concentration (MFC), can be used to assess the inhibitory and fungicidal potential of antifungal formulations. Such studies provide preliminary evidence of biological effectiveness and enable comparison between free and nanoformulated active compounds. Considering the established antifungal activity of allicin, its chemical instability, and the potential of β-cyclodextrin nanosponges to protect and deliver bioactive molecules, investigation of the antifungal activity of allicin-loaded β-cyclodextrin nanosponges is scientifically relevant. Therefore, the present study was undertaken to evaluate the in-vitro antifungal activity of allicin-loaded β-cyclodextrin nanosponges against selected fungal pathogens and to assess their potential as a nanoscale delivery system for enhancing the antifungal performance of allicin**.**

Material and Method

Material

Allicin was selected as the active pharmaceutical ingredient for the development of the topical nanosponge formulation. Ethyl Cellulose was used as the polymeric carrier. Polyvinyl Alcohol and Pluronic® F68 were used as stabilizing components during preparation of the nanosponges. Carbopol 940 was used as the gelling agent for the preparation of the topical gel, and Triethanolamine was used as the neutralizing agent.Dichloromethane was used as the organic solvent, as specified in the formulation method. Purified water was used as the aqueous vehicle

Preparation of Allicin-Loaded Nanosponges

Allicin-loaded nanosponges were prepared using different proportions of ethyl cellulose, polyvinyl alcohol, and Pluronic F68 by the emulsion solvent diffusion technique. The disperse phase was prepared by dissolving Allicin (24.32 mg) and the specified quantity of ethyl cellulose, as shown in Table 7.5, in 30 mL of dichloromethane. This organic phase was slowly added to a predetermined quantity of polyvinyl alcohol dissolved in 100 mL of the aqueous continuous phase containing Pluronic F68.The resulting emulsion was stirred at 1000 rpm using a magnetic stirrer for 2 h. Following the completion of the process, the formed Allicin nanosponges were collected by vacuum filtration and dried in an oven at 40 °C for 24 h. [9]

In Vitro Antifungal Activity off Alicin-Loaded Nanosponge Formulation

The in vitro antifungal activity of the optimized allicin-loaded nanosponge formulation was evaluated against the selected fungal strain using agar well diffusion assay, minimum inhibitory concentration (MIC), time-kill assay and minimum fungicidal concentration (MFC) studies. The antifungal activity of the optimized nanosponge formulation was compared with free allicin and an appropriate standard antifungal drug.

Test microorganism

The antifungal activity was evaluated against the selected fungal test organism, *Candida albicans*. The authenticated culture was obtained from Pravara Institute of Medical Science and maintained according to the recommended microbiological procedure. The organism was subcultured on Sabouraud Dextrose Agar (SDA) and incubated under appropriate conditions before preparation of the inoculum.For yeast susceptibility testing, standardized broth dilution methodology based on CLSI M27 was considered for MIC evaluation.

Preparation of fungal inoculum

A fresh culture of *Candida albicans* was used for preparation of the fungal inoculum. Well-isolated colonies were aseptically transferred into sterile physiological saline and thoroughly mixed to obtain a uniform suspension.The turbidity of the suspension was adjusted to the required standardized inoculum density using a McFarland standard or an equivalent validated method.For broth microdilution testing, the final inoculum should conform to the applicable standardized procedure. CLSI M27 describes standardized inoculum preparation and broth dilution methodology for yeasts.

Experimental groups

The following groups were included in the antifungal evaluation:

Table 1: Experimental groups for antifungal activity

Group	Treatment
Group I	Negative control/vehicle
Group II	Blank nanosponge
Group III	Free allicin
Group IV	Optimized Allicin-loaded nanosponge (PF7)
Group V	Standard antifungal drug

Agar well diffusion assay

The antifungal activity was initially evaluated using the agar well diffusion method. Sterile Sabouraud

Dextrose Agar plates were prepared and allowed to solidify. A standardized suspension of *Candida albicans* was uniformly inoculated onto the surface of the agar using a sterile swab.After uniform inoculation, wells of approximately 6 mm diameter were aseptically prepared using a sterile cork borer.A measured quantity of each test preparation was introduced into the respective wells. The plates were allowed to stand for a short period to facilitate diffusion of the test preparations into the agar matrix. The inoculated plates were then incubated 24 hours at a temperature of 37°C under suitable conditions for *Candida albicans*. After incubation, the plates were examined for the formation of clear zones surrounding the wells.

The diameter of the zone of inhibition (ZOI) produced by each test preparation was measured in millimetres using a calibrated ruler or Vernier caliper. The diameter of each inhibition zone was measured in two perpendicular directions**,** and the mean diameter was calculated. The antifungal activity of the formulations was assessed by comparing the mean zones of inhibition produced by free allicin, blank nanosponge, optimized PF7, vehicle control, and the standard antifungal drug.

Table 2: Treatment for agar well diffusion method for antifungal activity

Treatment	Concentration	Volume/well
Vehicle control	0 mg/mL	100 µL
Blank nanosponge	16 mg/mL	100 µL
Free allicin	1 mg/mL	100 µL
Optimize Allicin-loaded nanosponge (PF7)	16 mg/mL formulation containing 0.243 mg/mL allicin	100 µL
Standard antifungal	0.1 mg/mL (100 µg/mL)	100 µL

Calculation:

The diameter of the inhibition zone was measured in two perpendicular directions and the mean diameter was calculated.

$$\text{Mean zone diameter} = D1+D2/2$$

Where:

D1 = diameter measured in the first direction

D2 = diameter measured in the second direction

Minimum Inhibitory Concentration (MIC)

The MIC of free allicin and Optimized Allicin-loaded nanosponge (PF7) was determined using a broth dilution procedure based on the principles of the CLSI M27 reference method for yeast susceptibility testing. CLSI M27 is specifically intended for standardized broth dilution testing of yeasts including *Candida* species. Serial two-fold dilutions of the test preparations were prepared in the selected broth medium. The concentrations evaluated may be selected according to the preliminary susceptibility range obtained from the agar diffusion study. The standardized fungal inoculum was added to each test concentration. Growth and sterility controls were included. After incubation under the validated conditions, the tubes/wells were examined for visible fungal growth. The MIC was defined as the lowest concentration of the test preparation showing no visible growth compared with the appropriate growth control, according to the selected standardized procedure. CLSI emphasizes standardized media, inoculum preparation, incubation and endpoint determination for reproducible yeast susceptibility testing.

Table 3: The MIC activity of free allicin and Optimized Allicin-loaded nanosponge

Tube/Well	Concentration
1	1000 µg/mL
2	500 µg/mL
3	250 µg/mL
4	125 µg/mL
5	62.5 µg/mL
6	31.25 µg/mL
7	15.625 µg/mL
8	7.8125 µg/mL
9	3.906 µg/mL
10	1.953 µg/mL

Time-Kill Assay

A time-kill study was performed to evaluate the rate and extent of antifungal activity of free allicin and Optimized Allicin-loaded nanosponge (PF7) against *Candida albicans*. A freshly prepared standardized fungal inoculum was exposed to selected concentrations of the test preparations based on the experimentally determined MIC. The study was conducted using 1× MIC and/or 2× MIC, according to the experimental design. Samples were collected at predetermined time intervals of 0, 2, 4, 8, 12 and 24 hr**.** At each time point, an aliquot was withdrawn and appropriately diluted using sterile diluent. The diluted samples were plated onto suitable agar medium for viable fungal count determination. Following incubation, colonies were counted and expressed as CFU/mL and converted to log10.

The logarithmic reduction was calculated using:
Log reduction=log10(CFU/mL at 0 h)−log10 (CFU/mL at time t

Result

Formulation of Allicin-loaded nanosponges

Allicin-loaded nanosponges were successfully prepared by the emulsion solvent diffusion technique using ethyl cellulose as the matrix-forming polymer, polyvinyl alcohol as the stabilizing agent, and Pluronic F68 as a surfactant. The formulations were obtained as free-flowing, dry particles after vacuum filtration and drying at 40 °C for 24 h. The successful formation of nanosponges indicated that the selected combination of organic solvent, polymer, stabilizer, and surfactant was suitable for the preparation of the nanosponge system. The gradual addition of the dichloromethane phase containing Allicin and ethyl cellulose into the aqueous PVA–Pluronic F68 phase, followed by continuous stirring at 1000 rpm, resulted in the formation of a stable emulsion. Evaporation/diffusion of dichloromethane during stirring promoted precipitation of ethyl cellulose around the dispersed phase, resulting in the formation of Allicin-loaded nanosponges.

In Vitro Antifungal Activity Of Alicin-Loaded Nanosponge Formulation 1.

Agar well diffusion assay

The antifungal activity of free allicin and allicin-loaded nanosponge against *Candida albicans* was evaluated by measuring the zone of inhibition.The vehicle control and blank nanosponge showed no antifungal activity **(**0.00 ± 0.00 mm**).** Free allicin produced a zone of inhibition of 14.2 ± 0.6 mm, whereas the allicin-loaded nanosponge showed a significantly larger zone of 21.8 ± 0.8 mm**.** The standard antifungal showed 24.6 ± 0.5 mm. Thus, the allicin-loaded nanosponge exhibited approximately 53.5% greater inhibition than free allicin and demonstrated enhanced apparent antifungal activity against *Candida albicans*.

Table 4: Antifungal activity of allicin-loaded nanosponge against *Candida albicans* by agar well diffusion method

Treatment	Concentration	Volume/well	Zone of inhibition (mm), Mean ± SD
Vehicle control	0 mg/mL	100 µL	0.00 ± 0.00
Blank nanosponge	16 mg/mL	100 µL	0.00 ± 0.00
Free allicin	1 mg/mL	100 µL	14.2 ± 0.6
Optimized Allicin-loaded nanosponge (PF7)	16 mg/mL formulation containing 0.243 mg/mL allicin	100 µL	21.8 ± 0.8
Standard antifungal drug	0.1 mg/mL (100 µg/mL)	100 µL	24.6 ± 0.5

Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration of free allicin and Optimized Allicin-loaded nanosponge (PF7) was determined against *Candida albicans* using the broth dilution method. The MIC of free allicin was found to be 125 µg/mL, whereas the Optimized Allicin-loaded nanosponge (PF7) exhibited an MIC of 62.5 µg/mL. The standard antifungal drug showed an MIC of 31.25 µg/mL**.**

The allicin-loaded nanosponge demonstrated a two-fold reduction (50%) in MIC compared with free allicin, indicating enhanced inhibitory activity against *Candida albicans*. The lower MIC observed for the nanosponge formulation suggests that incorporation of allicin into the nanosponge system improved its antifungal efficacy under the tested conditions.

Table 5: Minimum inhibitory concentration (MIC) of free allicin and allicin-loaded nanosponge against *Candida albicans*

Test preparation	MIC (µg/mL)
Free allicin	125
Allicin-loaded nanosponge	62.5
Standard antifungal	31.25

Time-Kill Assay

The time-kill assay demonstrated a time-dependent reduction in the viable count of *Candida albicans* following treatment with free allicin and allicin-loaded nanosponge. The viable fungal count

decreased progressively with increasing exposure time for both treatments, whereas the untreated control showed progressive fungal growth. At 2 hr, free allicin reduced the viable count from 6.20 to 5.75 $\log_{10}$ CFU/mL, corresponding to a 0.45-log reduction, while the allicin-loaded nanosponge reduced the count to 5.30 $\log_{10}$ CFU/mL, corresponding to a 0.90-log reduction. At 8 h, the reductions were 1.75 $\log_{10}$ for free allicin and 3.00 $\log_{10}$ for the allicin-loaded nanosponge. After 24 h of treatment, the viable counts were 2.80 $\log_{10}$ CFU/mL for free allicin and 1.00 $\log_{10}$ CFU/mL for the allicin-loaded nanosponge, corresponding to reductions of 3.40 $\log_{10}$ and 5.20 $\log_{10}$, respectively, from the initial count of 6.20 $\log_{10}$ CFU/mL. In contrast, the untreated control increased from 6.20 to 7.20 $\log_{10}$ CFU/mL over 24 h.

Thus, the allicin-loaded nanosponge produced a greater time-dependent reduction in viable *Candida albicans* than free allicin, with the maximum reduction observed at 24 h. These findings indicate enhanced antifungal activity of the nanosponge formulation under the tested conditions.

Table 6: Effect of free allicin and allicin-loaded nanosponge on viable *Candida albicans* count

Time (h)	Free allicin ($\log_{10}$ CFU/mL)	Allicin-loaded nanosponge ($\log_{10}$ CFU/mL)	Control ($\log_{10}$ CFU/mL)
0	6.20	6.20	6.20
2	5.75	5.30	6.35
4	5.20	4.40	6.50
8	4.45	3.20	6.75
12	3.70	2.10	6.95
24	2.80	1.00	7.20

Table7: Log reduction in viable *Candida albicans* count relative to 0 h

Time (h)	Free allicin	Allicin-loaded nanosponge	Control
0	0.00	0.00	0.00
2	0.45	0.90	−0.15
4	1.00	1.80	−0.30
8	1.75	3.00	−0.55
12	2.50	4.10	−0.75
24	3.40	5.20	−1.00

Discussion :

Allicin-loaded nanosponges were successfully prepared by the emulsion solvent diffusion technique using ethyl cellulose, PVA, and Pluronic F68. Among the prepared formulations, PF7 containing 24.32 mg Allicin, 500 mg ethyl cellulose, 900 mg PVA, and 200 mg Pluronic F68 was selected as the optimized formulation based on its overall formulation characteristics.The optimized Allicin-loaded nanosponge demonstrated enhanced antifungal activity against *Candida albicans* compared with free Allicin. The formulation exhibited a higher zone of inhibition **(**21.8 ± 0.8 mm**)** than free Allicin **(**14.2 ± 0.6 mm**),** indicating improved antifungal efficacy following nanosponge incorporation. Furthermore, the MIC of the optimized formulation was reduced to 62.5 µg/mL**,** compared with 125 µg/mL for free Allicin, demonstrating a two-fold improvement in antifungal potency.

The time-kill study further confirmed the enhanced activity of the nanosponge formulation. The Allicin-loaded nanosponge produced a progressive reduction in viable *C. albicans* cells, reaching 1.00 $\log_{10}$ CFU/mL at 24 h, compared with 2.80 $\log_{10}$ CFU/mL for free Allicin. This enhanced activity may be attributed to improved dispersion and sustained availability of Allicin from the nanosponge matrix, facilitating greater interaction with fungal cells.Overall, incorporation of Allicin into nanosponges significantly improved its in-vitro antifungal performance, suggesting that the developed nanosponge system is a promising approach for enhancing the efficacy of Allicin against *C. albicans*.

Conclusion:

The present study demonstrated the successful development of Allicin-loaded nanosponges using the emulsion solvent diffusion technique **a**nd their potential for enhancing the antifungal activity of Allicin against *Candida albicans*. The optimized PF7 formulation exhibited a greater zone of inhibition **(**21.8 ± 0.8 mm) than free Allicin (14.2 ± 0.6 mm) and showed a two-fold reduction in MIC from 125 to 62.5 µg/mL. The time-kill study further confirmed the superior antifungal effect of the nanosponge formulation, producing a 5.20-$\log_{10}$ reduction in

viable fungal count after 24 h, compared with a 3.40-$\log_{10}$ reduction with free Allicin.These findings indicate that incorporation of Allicin into the nanosponge system can substantially enhance its in-vitro antifungal performance against *C. albicans*. The developed formulation therefore represents a promising nanocarrier-based approach for improving the delivery and antifungal efficacy of Allicin. Further studies addressing formulation stability, drug-release behavior, safety/cytotoxicity, and in-vivo antifungal efficacy are warranted to establish its potential for therapeutic application

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