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In vitro efficacy of antifungal activities either singly or loaded on nanomaterials, as new and novel drug delivery systems on the growth of Alternaria alternata and Botrytis fabae fungal pathogens

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Abstract: The present study aims to search for a novel nano-drug delivery systems through carrying out *in vitro* experiment that enables access of antifungal antibiotics to the targeting site of the fungal pathogens. To achieve this target, two antifungals; nystatin (NYS) and fluconazole (FLZ), were loaded on three different nanomaterials; chitosan nanoparticles (CSNPs), carbon nanotubes (CNTs) and solid lipid nanoparticles (SLNPs) to facilitate drug delivery to the fungal pathogens (*Alternaria alternata* and *Botrytis fabae*). The nano drug delivery systems were evaluated by determination of the efficient antifungal activity on the fungal pathogens comprising the tested drug delivery systems. The results obtained revealed that the tested strains were resistant to sole nanomaterials (CSNPs, CNTs and SLNPs) and sensitive to the loaded nanomaterials with either nystatin or fluconazole antifungals. The efficiency of the tested three drug delivery systems revealed that loaded solid nanoparticles (SLNPs) were the most efficient nano drug delivery systems for controlling the two fungal pathogens.

keywords: antifungals, fungi, nanomaterials, nystatin.

1.Introduction

Fungal antibiotics are among the most significant therapeutic breakthroughs in medical history. [1, 2]. Such antibiotics have improved the way we treat patients with fungal infections and have assisted in lowering the deaths and morbidity associated with microbial diseases [3]. Despite the existence of both conventional and contemporary antifungal medications, the frequency of mycotic infections is continually rising [4, 5]. This issue might be attributed to fungi's capacity to create multiple mechanisms for resistance to the current antimycotic treatments [6]. Additionally, the current antifungals have some flaws in their effectiveness, efficiency, toxicity, selectivity, resistance mechanisms and activity spectra. [7]. In this connection, the development of drug delivery systems relying on nanomaterials or nanoparticles (NPs) is a potential substitute for developing novel pharmaceutical formulations to effectively treat fungi and circumvent their multi-drug resistance [8].

The emergence of new, violent parasites and bacteria that are resistant to traditional antibiotics necessitates the development of novel therapeutic approaches that will overcome these pathologies' limits in treatment and the ineffectiveness of conventional antibiotics [9]. Nanostructured biomaterials, and nanoparticles in particular, possess special physicochemical characteristics, such as ultra-small and controlled size, strong reactivity, a high surface area to mass ratio, and functionalization structure. Due to these qualities, one can get around some of the drawbacks of conventional antimicrobial therapies and make it easier to administer antimicrobial medications [10].

Nanoparticles can be used to protect plants in two different ways: either directly as crop protection agents or as carriers for existing pesticides or other actives like double-stranded RNA (dsRNA) Nanoparticles can be applied to seeds, foliar tissue, or roots by spraying, soaking, or drenching them [11]. As carriers, nanoparticles can offer several advantages,

including longer shelf life, higher pesticide solubility in water, decreased toxicity, and increased site-specific uptake by the target pest [11]. Additional potential advantage of nanocarriers is an improvement in the activity and durability of nano pesticides and antifungal under environmental stressors (UV and rain), as well as large decrease in the number of applications, lowers toxicity and costs [12].

In view of the above, the present study aims to evaluate the efficacy of nystatin and fluconazole antifungals loaded on three different nanomaterials to overcome the unfavorable toxicity profiles and to be used as alternative treatments for *Alternaria alternata* and *Botrytis fabae* pathogenic fungi.

2. Materials and methods

MATERIALS

Fungal isolates: pure identified isolates of *Botrytis fabae* and *Alternaria alternata* pathogenic fungi were obtained from the seed pathology and tissue culture laboratory, faculty of agriculture, Mansoura, Egypt.

Nanomaterials: chitosan nanoparticles (CSNPs) prepared according to [13, 14], carbon nanotubes (CNTs) prepared following the method of [15, 16]. Solid lipid nanoparticles (SLNPs) were prepared following the hot homogenization method with special modification and innovation according to [17].

Chemicals and antifungals: Nystatin and fluconazole antifungals were obtained from Sigma Aldrich, Germany. All chemicals used were of analytical grade.

METHODS

Minimum fungicidal concentration of nystatin and fluconazole antifungals (MFC)

The minimum fungicidal concentration (MFC) was detected by subculturing the tested dilutions that inhibited the growth of the tested pathogens in the MFC assay. The concentrations of 25, 50 and 75 of µg/ml of nystatin and fluconazole antifungals were inoculated into a potato dextrose agar (PDA) media pre-cultured with *Alternaria alternata* and *Botrytis fabae* pathogens. The agar plates were incubated at 25˚C for 6 days [18].

In Vitro antifungal activity of the prepared nanomaterials either alone or loaded with nystatin and fluconazole drugs

Assay of the antimicrobial activity of the prepared nanomaterials either alone or loaded with nystatin and fluconazole drugs was determined by cup plate method according to [19]. About 100 µl of selected antifungal fractions, nanomaterials and selected antifungal fractions loaded on the nanomaterials were added in 8 mm pore of agar plates previously spread with an inoculum of the pathogenic fungi on the surface. Inhibition zones were measured in mm after 6 days of the incubation at 25˚C.

Statistical Analysis

All experimental tests were performed in triplicates on the same time and the statistical analysis of experimental data was done using SPSS version 23. The results were analyzed statistically using one-way analysis of variance (ANOVA) with statistically significant differences at *P values $\geq$ 0.05.

3. Results and Discussion

Minimal fungicidal concentration of nystatin and fluconazole antifungals on the fungal pathogen

The three different concentrations of antifungal antibiotics (nystatin and fluconazole) showed variable broad activity against the growth of *Alternaria alternata* and *Botrytis fabae* pathogens (figures 1 and 2, tables 1 and 1). The two fungal pathogens were most sensitive at concentration of 50 µg/ml of both antifungal antibiotics (nystatin and fluconazole). This concentration showed optimum antibiotic activity.

Figure 1: Effect of different concentrations of nystatin antifungal on *Botrytis fabae*

Table 1: Antifungal potential of nystatin against *Botrytis fabae* pathogenic fungus. The values (*) are significantly different from control at P ≥ 0.05

Treatment	Zone of Inhibition (mm)	% Inhibition
Control	8	–
NYS 25	20*	150.0
NYS 50	22*	175.0
NYS 75	17*	112.5

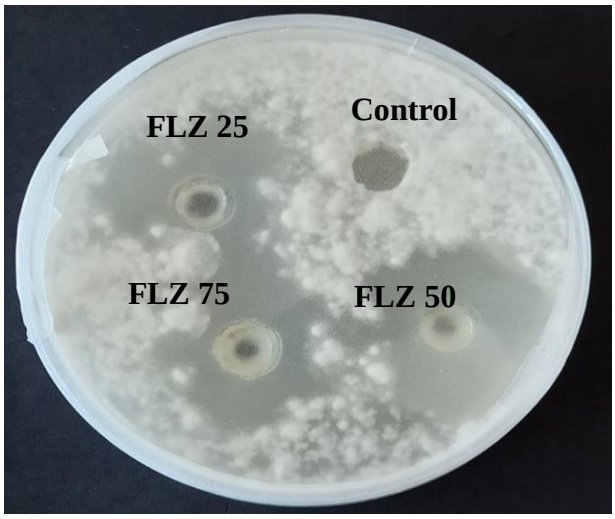

Figure 2: Effect of different concentrations of fluconazole antifungal on *Botrytis fabae*

Table 2: Antifungal potential of fluconazole against *Botrytis fabae* pathogenic fungus. The values (*) are significantly different from control at P ≥ 0.05

Treatment	Zone of Inhibition (mm)	% Inhibition
Control	8	–
FLZ 25	25 *	212.5
FLZ 50	28*	250.0
FLZ 75	23*	187.5

Effect of nanomaterials either singly or loaded with nystatin and fluconazole antifungals as drug delivery systems on the growth of *Alternaria alternata* and *Botrytis fabae* pathogens

Administration of nystatin and fluconazole antifungal antibiotics into the culture media of *Alternaria alternata* and *Botrytis fabae* pathogens induced variable inhibition zones of the growth of the pathogens (figures 3-6 and tables 3-6). The following sequence of treatments (SLNPs+FLZ > CSNPs+FLZ > FLZ > CNTs+FLZ > SLNPs > CNTs = CSNPs) was displayed with respect to inhibition zones of fluconazole antifungal on *Alternaria alternata* growth (figure 3 and table 3).

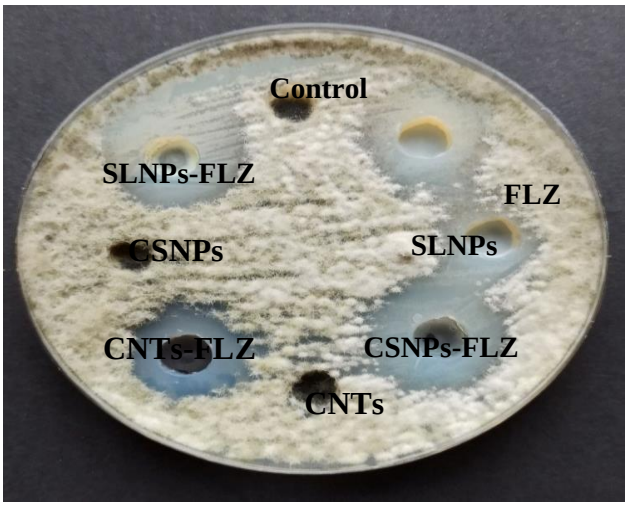

Figure 3: Antifungal activity of fluconazole either singly or loaded on CSNPs, CNTs and SLNPs on culture medium of *Alternaria alternata*.

Table 3: Antifungal potential of fluconazole either singly or loaded with CSNPs, CNTs and SLNPs against *Alternaria alternata*. The values (*) are significantly different from control at P ≥ 0.05.

Treatment	ZoneofInhibition (mm)	% Inhibition as percent of control	% Inhibition as percent of drug singly	% Inhibition as percent of nanostructure singly
Control	8	-	-	-
FLZ	20*	150.0	-	-
SLN	14*	75.0	-30.0	-
SLNPs-FLZ	25*	212.5	25.0	78.6
CSNPs	8	0.0	-60.0	-
CSNPs-FLZ	23*	187.5	15.0	187.5
CNTs	8	0.0	-60.0	-
CNTs-FLZ	18*	125.0	-10.0	125.0

Loading of nystatin antifungal with either SLNPs, CNTs and CSNPs as drug delivery system and administration of nano systems into the culture media of *Alternaria alternata* pathogen maintained variable inhibition zones arranged in the following sequence (SLNPs+NYS > NYS > CSNPs+NYS > SLNPs > CNTs+NYS > CNTs = CSNPs) (figure 4 and table 4).

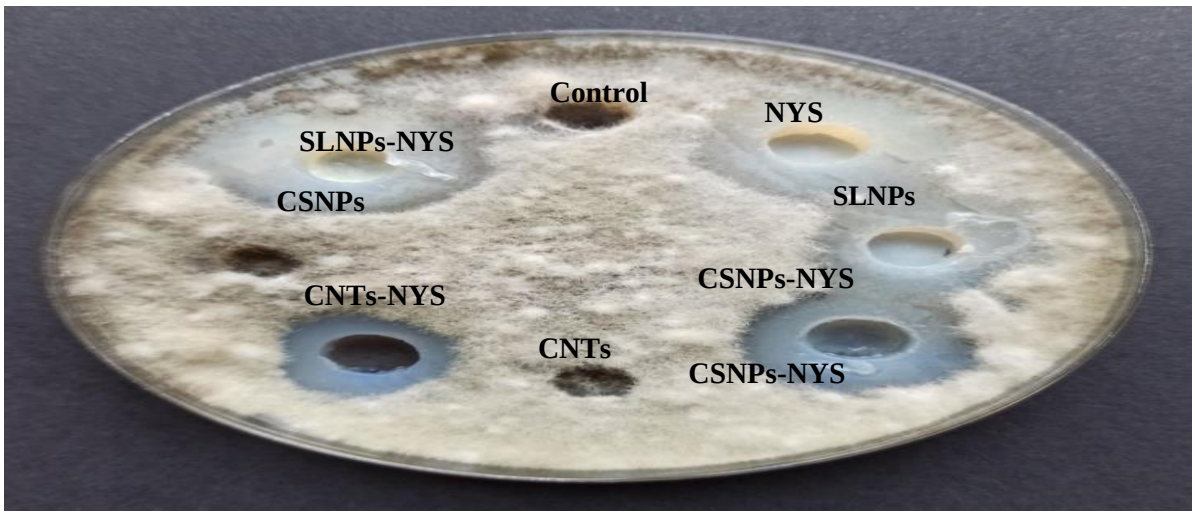

Figure 4: Antifungal activity of nystatin either singly or loaded with CSNPs, CNTs and SLNPs on culture medium of *Alternaria alternata*.

Table 4: Antifungal potential of nystatin either singly or loaded with CSNPs, CNTs and SLNPs against *Alternaria alternata*. The values (*) are significantly different from control at $P \geq 0.05$.

Treatment	Zone of Inhibition (mm)	% Inhibition as percent of control	% Inhibition as percent of drug singly	% Inhibition as percent of nanostructure singly
Control	8	-	-	-
NYS	20*	150.0	-	-
SLNPs	15*	87.5	-25.0	-
SLNPsNYS	24*	200.0	20.0	60.0
CSNPs	8	0.0	-60.0	-
CSNPsNYS	17*	112.5	-15.0	112.5
CNTs	8	0.0	-60.0	-
CNTs-NYS	14*	75.0	-30.0	75.0

On the other hand, the data herein reported that the activity of SLNPs, CNTs and CSNPs either singly or in combination against the growth of *Botrytis fabae* induced significant results as nano drug delivery systems (figures 5 and 6, tables 5 and 6). The following sequences of treatments (SLNPs+FLZ > FLZ > SLNPs > CSNPs+FLZ > CNTs+FLZ = CNTs = CSNPs) and (SLNPs+NYS > NYS > CSNPs+NYS > SLNPs > CNTs+NYS > CNTs = CSNPs) were, in general, displayed with respect to evaluation of fluconazole and nystatin antifungals, respectively, for *Botrytis fabae* fungal pathogen.

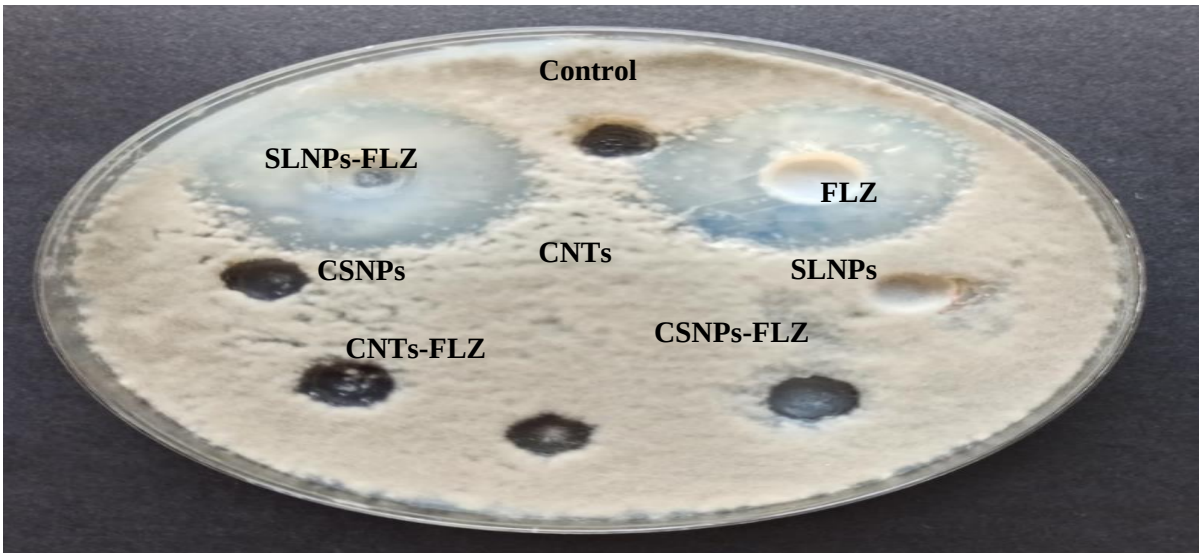

Figure 5: Antifungal activity of fluconazole either singly or loaded with CSNPs, CNTs and SLNPs on culture medium of *Botrytis fabae*.

Table 5: Antifungal potential of fluconazole either singly or loaded with CSNPs, CNTs and SLNPs against *Botrytis fabae*. The values (*) are significantly different from control at P ≥ 0.05.

Treatment	Zone of Inhibition (mm)	% Inhibition as percent of control	% Inhibition as percent of drug singly	% Inhibition as percent of nanostructure singly
Control	8	-	-	-
FLZ	25*	212.5	-	-
SLNPs	17 *	112.5	-32.0	-
SLNPsFLZ	28*	250.0	21.7	64.7
CSNPs	8	0.0	-68.0	-
CSNPsFLZ	12*	50.0	-52.5	50.0
CNTs	8	0.0	-68.0	-
CNTs-FLZ	8	0.0	-68.0	0.0

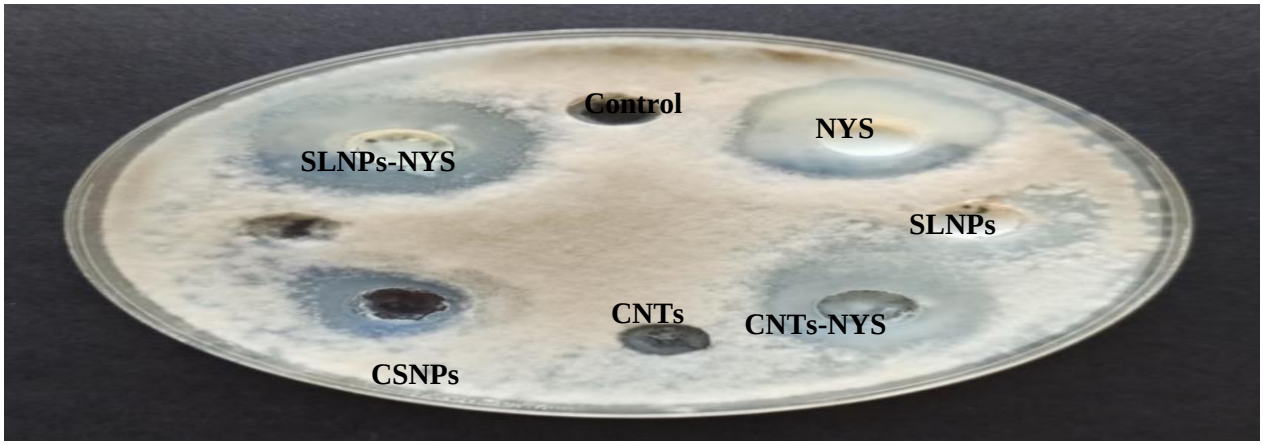

Figure 6: Antifungal activity of nystatin either singly or loaded with CSNPs, CNTs and SLNPs on culture medium of *Botrytis fabae*.

Table 6: Antifungal potential of nystatin either singly or loaded with CSNPs, CNTs and SLNPs against *Botrytis fabae*. The values (*) are significantly different from control at P ≥ 0.05.

Treatment	Zone of Inhibition (mm)	% Inhibition as percent of control	% Inhibition as percent of drug singly	% Inhibition as percent of nanostructure singly
Control	8	-	-	-
NYS	23*	187.5	-	-
SLNPs	14*	75	-39.1	-
SLNPsNYS	25*	212.5	8.7	78.6
CSNPs	8	0.0	-65.2	-
CSNPsNYS	19*	137.5	-17.4	137.5
CNTs	8	0.0	-65.2	-
CNTs-NYS	11*	37.5	-52.2	37.5

Perusal of the data revealed three main nanodrug delivery strategies: strategy A (SLNPs+FLZ; A1and/ or SLNPs+nys; A2), strategy B (CSNPs+FLZ; B1 and/ or CSNPs+NYS; B2) and strategy C (CNTs+FLZ; C1 and/ or CNTs+NYS; C2). It was found that strategy A > strategy B > strategy C as nanodrug delivery efficiency. On the other hand, the following sequence of all strategies

(strategy A > strategy B > strategy C) and (SA1 > SB1 > SC1) and (SA2 > SB2 > SC1) was displayed with respect to efficiency as nanodrug delivery systems. It is of interest in the present novel work to mention that the best nanodrug delivery strategy for treatment of fungal pathogens *Alternaria alternata* and *Botrytis fabae* was found with the strategy of solid lipid nanoparticles loaded with fluconazole antifungal followed by strategy of solid lipid nanoparticles loaded with nystatin antifungal.

The present research focuses on the development of experimental trials searching

for nanomaterial systems that enable us to enhance and restore the antibiotic activity for drug-resistance organisms. Adjuvant substances are important in enhancing the immunogenicity and efficacy of a medicine, particularly antibiotics and vaccines. Recently, the research on NPs has cleared the path for the development of more effective and efficient medicines and vaccines. Both inorganic and organic adjuvants have lately been investigated and used in NPs size. They have proven to be more advantageous than macromolecular forms in that they are less expensive and simpler to create. They have been found to have tremendous significance in entrapping and binding biomolecules of medicinal and veterinary interest. Additionally, it has been discovered that nanoscale formulations greatly improve the bioavailability of the biomolecules. Therefore, such nanoparticles have significant impact on a variety of sectors, including gene therapy, adjuvants, and drug delivery systems [20].

The results obtained (see tables 1 and 2) in the present study indicated that the tested antifungals; nystatin and fluconazole have showed well antimicrobial activity against *Alternaria alternata* and *Botrytis fabae* pathogenic fungi with most effective concentration of 50 µg/ml. The present results tend to suggest that the tested antifungals have a strong activity on pathogenic fungi [21]. As shown in figures 3-6, optimum concentration (50 µg/ml) of nystatin and fluconazole antifungals either singly or loaded with CSNPs, CNTs and SLNPs exhibit variable significant effect on the growth of the fungal pathogens; *Alternaria alternata* and *Botrytis fabae*. The valuable and new data obtained in the present study (figures 3 and 5, tables 3 and 5) showed that as compared with the antibiotic activity alone on the growth of *Alternaria alternata* and *Botrytis fabae* fungal pathogens, administration of CNTs, CSNPs and SLNPs loaded with fluconazole antifungal as nano drug delivery systems led to variable increases and decreases in its activity by a ratio of –10 %, +15 % and +25 % for CNTs, CSNPs and SLNPs, respectively for *Alternaria alternata* and by a ratio of –68 % for CNTs, -52.5 % for CSNPs and +21.7 % for SLNPs in case of *Botrytis fabae*. Thus, SLNPs loaded with fluconazole might be considered as an effective nano drug delivery system for inhibition of the growth of *Alternaria alternata* and *Botrytis fabae* pathogenic fungi.

On the other hand, inclusion of nystatin antifungal singly into culture media of *Alternaria alternata* and *Botrytis fabae* induced inhibition in the growth of the fungi under study by a ratio of +150 % for *Alternaria alternata* and +187.5 % for *Botrytis fabae*. Loading of nystatin onto the three different nanomaterials CNTs, CSNPs and SLNPs and their inclusion into the culture media of the two fungi as drug delivery systems showed different increases and decreases in the activity of the nano drug delivery systems by ratios of –30 %, -15 % and +20 % for CNTs, CSNPs and SLNPs, respectively for *Alternaria alternata* and by ratios of -52.5 % for CNTs, -65.2 % for CSNPs and +8.7 % for SLNPs in case of *Botrytis fabae* (see figures 4 and 6, tables 4 and 6).

Thus, the following sequence of nano drug delivery systems (SLNPs+FLZ > CSNPs+FLZ > CNTs+FLZ) was displayed with respect to maximum fluconazole antifungal activity for both *Alternaria alternata* and *Botrytis fabae* (figures 3 and 5, tables 3 and 5). Furthermore, the following sequence of treatments (SLNPs+NYS > CSNPs+NYS > CNTs+NYS) was shown maximum for nystatin drug activity against the two tested fungi (figures 4 and 6, tables 4 and 6).

Of interest for this connection, [22, 23, 24] reported that nanoparticles formulations can prolong the half-life of the antibiotic payload and serve as a sustained release system, reduce the toxicity of antibiotics, and improve their pharmacokinetics, reducing the frequency of administration and improving the therapeutic index [25]. There are three types of mechanisms of antimicrobial action of nano drug delivery systems that include reduction of oxidative stress, release of metal ions or non-oxidative mechanisms which can occur simultaneously [26]. The results of the present study are in good agreement with [27] whose results supported the use of alginate-based drug delivery system and nano systems for drug delivery as carriers for antifungal antibiotics for drug toxicity reduction and control of the

fungal infection in the in vivo model of pathogenic fungi. Additionally, our results revealed that unloaded CSNPs and CNTs showed no antimicrobial activity against *Alternaria alternata* and *Botrytis fabae* fungal pathogens. These results are in agreement with those obtained by [28] who found that *Aspergillus niger* fungus was resistant to CSNPs.

In conclusion, the results obtained herein support the use of nano-based drug delivery systems as carriers for fluconazole and nystatin antifungal antibiotics, increasing their activity spectra as compared to the free drug and controlling the fungal infection in the mycelial growth of *Alternaria alternata* and *Botrytis fabae* plant pathogenic fungi. Furthermore, SLNPs are very attractive nano colloidal carrier system for foliar spray application on plants due to their various desirable effects on plant body besides the characteristic of a colloidal carrier system. They are well suited for use on infected foliar parts of the plant because they are based on non-toxic lipids.

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