



Antimicrobial effect of chitosan–silver–copper nanocomposite on *Candida albicans*

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Abstract

Candida is a common yeast in opportunistic fungal diseases around the world and is usually colonized on the skin and mucosal membranes. The purpose of this study was to synthesize chitosan–silver–copper nanocomposite and to investigate its antifungal effects on *Candida albicans*. Silver, copper and chitosan nanoparticles were synthesized individually. Then, copper–silver–chitosan nanocomposite was synthesized. These nanoparticles are approved by transmission electron microscope, and nanocomposite structure was also confirmed by scanning electron microscope. Then, the minimum inhibitory concentrations and minimum fungicidal of these nanostructures were examined on *C. albicans*. The results of this study indicate that the properties and effects of the investigated nanocomposite are comparable to amphotericin B as standard material. The results show that this effect was higher for copper–silver–chitosan nanocomposite than for other nanoparticles studied. Antifungal effect of copper nanoparticles and chitosan nanoparticles was not established separately, but it was found that their composition had antifungal effects that were effective. The combination of nanoparticles of chitosan with silver has been shown to have some antifungal effects. The most antifungal effect for the nanoparticles studied is related to copper–silver–chitosan nanocomposite and, which has had a significant effect on the growth of *C. albicans* in the laboratory environment compared to other nanoparticles.

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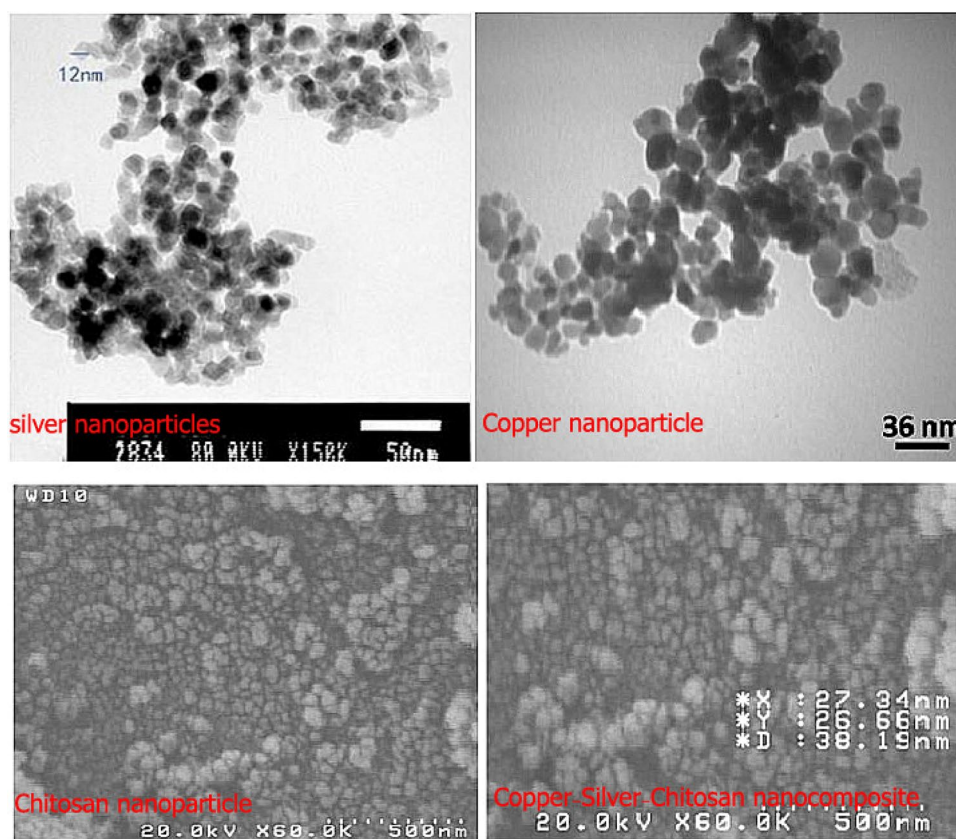
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Graphic abstract



Keywords *Candida albicans* · Chitosan · Silver · Copper · Nanocomposite

Introduction

Candida is a common yeast in opportunistic fungal diseases around the world and is usually colonized on the skin and mucosal membranes. *Candida* is one of the members of the normal flora of skin, mouth, vagina, and stool and is opportunistic [1]. In nature, especially are found on leaves of plants, water, and soil. *Candida albicans* is a pleomorphic mold that is the normal flora of the human body and animals [2]. Although *C. albicans* is the most common cause of invasive candidiasis and two-thirds of all *Candida* species isolated from these patients worldwide, other *Candida* species such as *Candida cruzi*, *Candida tropicalis*, and *Candida parapsilosis* are also pathogenic agents of invasive candidiasis [3, 4].

In addition to cutaneous and mucosal infections, these opportunistic pathogens in immunocompromised people can create a chronic or acute invasive infection that can be limited to an organ or as a diffuse disease [5–7]. These infections, which in most cases lead to death, are known as invasive candidiasis. One of the most important places

for microorganisms to penetrate the human body is the oral cavity. *C. albicans* are one of the most common microbial agents that live in the normal mucus and cause disease in susceptible individuals. Important factors in the *C. albicans* pathogenicity include adhesion, secretion of proteinase, deformation from yeast to hyphae, and biofilm formation [8].

At the moment, the initial diagnosis of this disease is difficult and often occurs by observing symptoms and clinical signs that are usually non-specific and cultivating, which is often negative or, if positive, very late, positive. Serologic examination for finding *Candida* antibodies and antigens in the circulation shows a variable sensitivity and specificity [9]. The use of serologic tests to find antibodies against *Candida* antigens, due to their normal flora colonization at mucosal surfaces, reduces its significance. In addition, *Candida* species antigens are often rapidly cleared from the circulation, which reduces the sensitivity of these tests to detect invasive candidiasis. Additionally, the use of serologic tests to find antibodies to *Candida* is very limited due to the inadequacy of the humoral immune system in immunocompromised people. Pathologic studies have high sensitivity and specificity, but in



patients with thrombocytopenia, the biopsy is associated with severe bleeding problems [10].

Fungal infections caused by *Candida* species and the increasing prevalence of Azole-resistant strains in immunocompromised patients are very important. The toxicity of the drugs used, the resistance to these fungi and the problems caused by drug interactions, necessitate the use of more effective drugs and less toxicity [11]. Today, in addition to trying to make effective chemical treatments and probiotic therapy for the treatment of candidiasis, many metal nanoparticles have been studied in the treatment of this disease and have received significant results [12–14]. Among these nanoparticles, silver, copper, zinc oxide, graphene and polymer nanoparticles, including chitosan, can be mentioned. In the meantime, alloy nanoparticles, silver nanoparticles, iodine, and copper have also been studied for their effects on different microorganisms [15–18].

Nanoparticles prepared with chitosan and derivatives of these nanoparticles typically possess a positive surface charge and mucoadhesive properties such that can adhere to mucus membranes and release the drug payload in a sustained release manner. Chitosan-based NP has various applications in non-parenteral drug delivery for cancer treatment, drug delivery, gastrointestinal and pulmonary diseases [19]. Chitosan nanoparticles (CS-NPs) were obtained by ionic gelation. The SEM revealed the compatibility of chitosan as well as Ag-NPs, were inserted in the polymer matrix and dispersed on the surfaces of the prepared bionanocomposites [20, 21]. Silver nanoparticles, often described as silver, are composed of a large percentage of silver oxide, due to the high ratio of silver atoms in the bulk surface. Depending on the intended application, numerous forms of nanoparticles can be made. Spherical silver nanoparticles are commonly used [22]. Silver nanoparticles were displayed good antibacterial properties contrary to Gram-positive and Gram-negative bacteria [23]. Copper nanoparticles like many other forms of nanoparticles, a particle can be formed by natural processes or by chemical synthesis [24].

Limited antifungal drugs are used to treat candidiasis [25]. Among the new antimicrobial agents, special attention has been paid to nanoparticles. Nanoparticles have a higher level than other antifungal agents, and in addition, they have a better penetration in tissues and cells. Some metal oxide nanoparticles, such as zinc oxide, copper oxide, and silver oxide, have many antimicrobial effects [15, 26]. The purpose of this study was to synthesize chitosan–silver–copper nanocomposite and to investigate its antifungal effects on *C. albicans*.

Materials and methods

Synthesis of silver nanoparticles

To prepare silver nanoparticles, 0.5 g of silver nitrate (Merck Co., Germany) was added to 100 ml distilled water. After complete dissolution, the reducing agent sodium borohydride (Merck, Germany) was added at a speed of 5000 rpm (magnetic stirrer, IKA, Germany). The silver resuscitation and stirring solutions were continued for 30 min. During this time, the color of the solution changed from clear to brown and silver nanoparticles were formed in this solution [27, 28]. Silver nanoparticles synthesized to provide an electron microscope image (Tunneling Electron microscope, TEM, Philips, The Netherlands) were stored in the Falcon tube. Before taking an electron microscope imaging, a solution containing silver nanoparticles was thoroughly sonicated by an appropriate sonicator (Model 55743-Fritsch, Germany). It was well exposed to supersonic waves, so that the accumulated particles could be well separated.

Synthesis of copper nanoparticles

Amount of 0.5 g copper chloride was added to 100 ml distilled water. After dissolving the copper salt, the solution turns bright blue. At this stage, the sodium borohydride reduction agent was slowly added while the solution was stirred at about 5000 rpm (magnetic stirrer, IKA, Germany). In this way, while producing copper nanoparticles, the solution was changed to a dark blue color [29].

Synthesis of chitosan nanoparticles

To prepare nanoparticles of chitosan, 1 g of pure chitosan was dissolved in 100 ml of distilled water containing 1% acetic acid. Stirring was then continued for 15 min until a clear solution was obtained using a magnetic stirrer with a 5000 rpm (magnetic stirrer, IKA, Germany). The solution was placed in a sonicator apparatus (Model 55743-Fritsch, Germany) for 20 min and then slowly added to the 2 wt% glutaraldehyde solution while the solution was stirred on a magnetic stirrer. After adding glutaraldehyde, stirring was continued for 2 h in dark conditions [30].

Synthesis of chitosan–silver–copper nanocomposite

With the aim of synthesizing nanocomposite, the amount of 0.1 g of chitosan was dissolved in 100 ml of water with an ultrasonic device (Model 55743-Fritsch, Germany), pH adjusted with 1% acetic acid solution in pH 3 (Mettler Toledo Digital pH Meter, Swiss). The solution is exposed



to ultrasonic waves for 15 min (Model 55743-Fritsch, Germany). The magnetic stirrer was then placed at 5000 rpm (magnetic stirrer, IKA, Germany). In the meantime, colloids of synthesized silver nanoparticles and copper nanoparticles were added. Stirring continued for another 15 min. The diluted glutaraldehyde solution was then added dropwise to the solution, while the solution was stirred at 7000 rpm [31].

Determination of synthesized nanocomposite

An FT-IR technique was used to confirm the binding of chitosan and glutaraldehyde, and the synthesis of the nanocomposite and scanning electron microscopy (Philips XL30 scanning microscope, Philips, The Netherlands) was used to determine the size and morphology of the synthesized nanocomposite.

The FT-IR spectrum of copper–silver–chitosan nanocomposite was performed using Fourier-transform infrared spectroscopy in the range of 0–4000 cm^{-1} wavelength. (Shimadzu FT-IR 4300 spectrometer, Japan).

Candida albicans culture and preparation of nanoparticles

To study the effect of nanoparticles on *C. albicans*, the standard strain of *C. albicans* was used in the culture medium of Sabouraud dextrose agar (SDA) containing chloramphenicol (Thermo Fisher Scientific Inc. Code CM0041). The inoculum was incubated at 37 °C for a period of between 24 and 48 h. The colonies were diluted with distilled water to create opacities at 0.5 McFarland. The concentration of yeast at this dilution level was $1\text{--}5 \times 10^6$ CFU/ml. Nanoparticles were prepared in a 5% acetic acid medium in *Candida* culture medium. For this purpose, copper, silver, chitosan nanoparticle, and copper–silver–chitosan nanocomposite were weighted individually on an Analytical Balance (Sartorius, Germany) at 50 milligrams and diluted with *Candida* medium. These concentrations were finally measured at concentrations of 10, 20, 40, 80, 160, 320, 640, 1280 and 2560 mg/l. Preparation of the control medium was also done in a similar manner with respect to 0% concentration for the nanoparticle.

To investigate the antifungal activity of the nanoparticles studied, a Sabouraud dextrose agar medium containing chloramphenicol was prepared. Then continued for 48 h at 37 °C to incubate the necessary colonies of the fungus. This solution was added to the culture medium at 100 ml at three levels of previously prepared nanoparticles, and incubation was performed. Subsequently, a newly prepared *C. albicans* concentration of 0.5 McFarland was grown in the presence of nanoparticles and nanocomposite. After 24–24 h incubation, the lowest concentration of nanocomposite that *C.*

albicans were unable to grow was considered as the concentration of growth inhibitor.

MIC determination of nanoparticles

Determination of minimum inhibitory concentration (MIC) for 48 h incubation at 37 °C was performed properly (M27A2 CLSI document). To prevent sedimentation and accumulation of nanoparticles and to have a homogenous suspension of nanoparticles, each of the nanoparticles was introduced into a 5% acetic acid solution and placed in a sonicator device for 15 min before being used in a *Candida* medium.

Investigating the effect of nanoparticles on culture media

To investigate and compare the effect of the nanoparticles studied on *C. albicans*, 200 μl of the medium solution was added with 200 μl of diluted fungal suspension to a ratio of 0.5 McFarland to 200 μl wells of the nanoparticles. The treatments in this section include wells containing copper–silver–chitosan nanoparticles and copper–silver–chitosan nanocomposite, which are well exposed to ultrasonic waves before they enter the wells. In addition to the treatment samples, a control sample was also considered along with each of the treatments mentioned. Also, to investigate and evaluate the negative effect of acetic acid on the growth of the fungus, negative control samples were considered with a suitable acetic acid dilution. Also, in a separate column, positive control samples were prepared by adding the appropriate dilution series of amphotericin B.

Determine MFC nanoparticles

The contents of each of the wells were examined. If the well content is transparent, it means that there is no growth of the fungus in it. In this case, the wells were completely homogeneous with the aid of a micro-pipette, and each well was re-cultivated by sabouraud dextrose agar as three plates of 90×15 mm. To prevent any antifungal transfer, dilutions were introduced into the agar plate and then allowed to immersion. After the plate was dried, the cells were removed from the medium containing the drug. All plates were placed at 37 °C for 48 h. If the *Candida* fungus developed in the plate, the concentration of the nanoparticle equivalent in the microplate was recorded as MFC.

Determination of MIC, MFC nystatin as a positive control agent

The dilutions used for the determination of nystatin are 0.4–0.25 $\mu\text{g/ml}$. To determine nystatin MIC, 1 ml of the



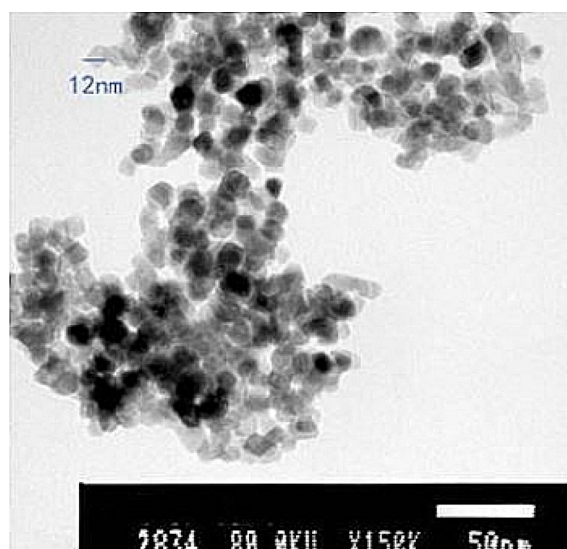


Fig. 1 TEM image of the synthesized silver nanoparticles

broth medium is added to sterile test tubes, and then the dilutions of the desired nystatin are added to the tubes. Then, 50 μ g of *Candida* suspension were added to the tubes, and the tubes were incubated at 30 °C for 48 h. Upon completion of this period, the MIC will be determined. To confirm the MIC results, the minimum nystatin concentration was also determined. For this test, 100 μ l of each of the MIC concentrations and above is added to the sterile plates containing the SDA medium. They are then incubated at 30 °C for 48 h. The lowest concentration in which the growth of *Candida* colony is not observed is considered as MFC (M38-A2 CLSI document).

Results

The electron microscope images of copper, silver and chitosan nanoparticles, synthesized are shown in Figs. 1, 2 and 3 and structure of synthetic nanocomposite is shown in Fig. 4 a spherical morphology for copper and silver nanoparticles with in very low size lower than 50 nm and 36 nm can be observed in scale bar of TEM Figs. 1 and 2, respectively. According to these microscopic images, the average particle size for copper and silver nanoparticles were 12 nm and 20 nm, respectively.

A semi-amorphous and slightly spherical morphology can be observed for chitosan nanoparticles and synthetic nanocomposite in SEM images in Figs. 3 and 4, respectively. As it is obvious from these images, the size and morphology of chitosan and nanocomposite were nearly similar and lower than 50 nm. As it was revealed from Fig. 4, the chitosan nanocomposite was dispersed in the solution homogeneously. According to the microscopic images that are presented in

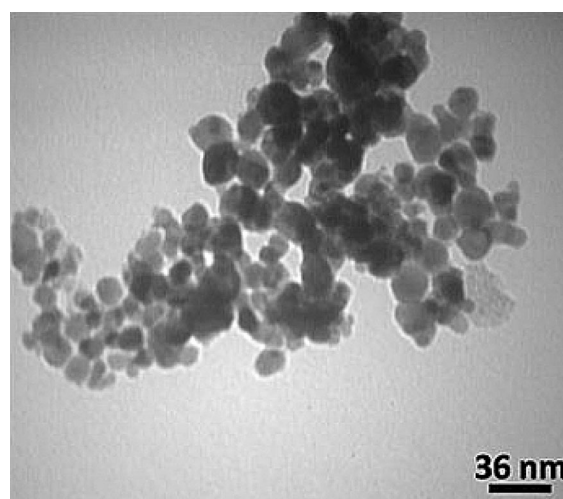


Fig. 2 TEM image of the synthesized copper nanoparticle

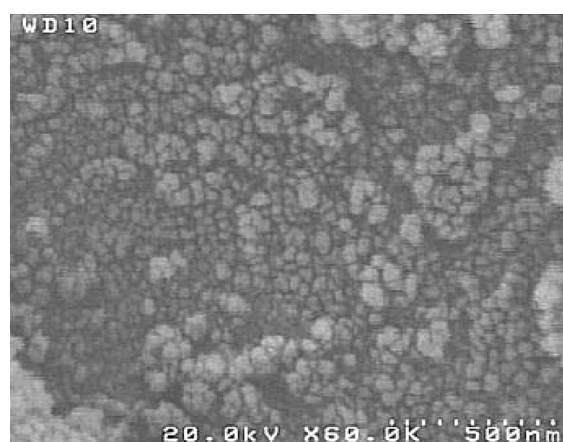


Fig. 3 SEM image of the synthesized nano-chitosan nanoparticle

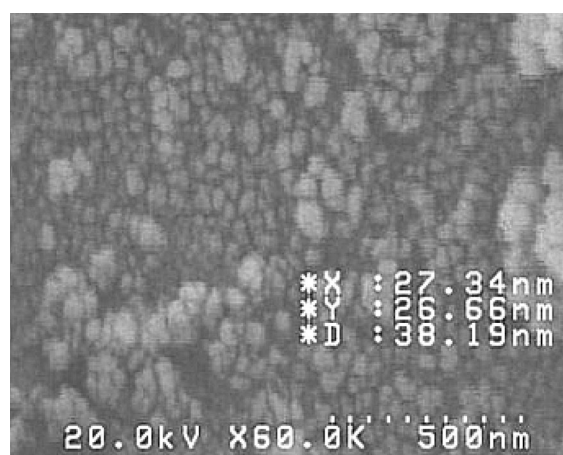


Fig. 4 SEM electron microscope image (Philips XL30 scanning microscope, Philips, The Netherlands) of the synthesized copper-silver-chitosan nanocomposite



Figs. 3, 4, the average size for chitosan and synthetic nanocomposite were 40 and 55 nm, respectively.

Also Fig. 5 is FT-IR of copper–silver–chitosan nanocomposite. As shown in Fig. 5, the absorption band in the region of 550 cm^{-1} is related to the Ag–O tensile vibration, which indicates the presence of silver in the nanocomposite structure. The tensile strips observed in the 1080 cm^{-1} and 1100 cm^{-1} regions are related to the tensile vibrations of C–O–C in the chitosan structure. The tensile band observed in the spectrum in the 1360 cm^{-1} area is also related to the tensile vibration of the C–O functional group from the alcoholic factors present in the chitosan structure.

MIC determination results

Table 1 presents the results of determining the lowest concentration of nanoparticles that *C. albicans* cannot grow. These results are for 48 h incubation at $37\text{ }^{\circ}\text{C}$, which is done visually and recorded. In order to investigate the synergistic or defective effects of the nanoparticles studied on *C. albicans*, 1:1 suspension from copper–silver–chitosan

nanoparticles were used in treatment No. 4, 5, and 6. Due to some antibacterial and antifungal characteristics of silver, copper and chitosan that were reported individually in some previous reports, in this study, the effects of these nanoparticles were considered as the potential controlling agents in antifungal investigating dosing. So for the same effect for the simultaneous effect of the three nanoparticles studied, the ratio of 1:1:1 nanoparticle was used in treatment No. 7.

MFC determination results

The results for the lowest concentrations of nanoparticle that exhibit antifungal characteristics are presented in Table 1. If the contents of the well were transparent, the result of the test indicated that there was no growth of the fungus was observed. The results were evaluated for each plate at $37\text{ }^{\circ}\text{C}$ and after 48 h. If the *Candida* had a growth in the plate, the equivalent concentration of the nanoparticles in the microplate was considered as MFC. As the obtained results are clearly present in Table 1, copper/silver nanoparticles, copper/chitosan nanoparticles and silver/chitosan nanoparticles individually revealed lower inhibitory and antifungal effects in comparison with silver/copper chitosan nanocomposite.

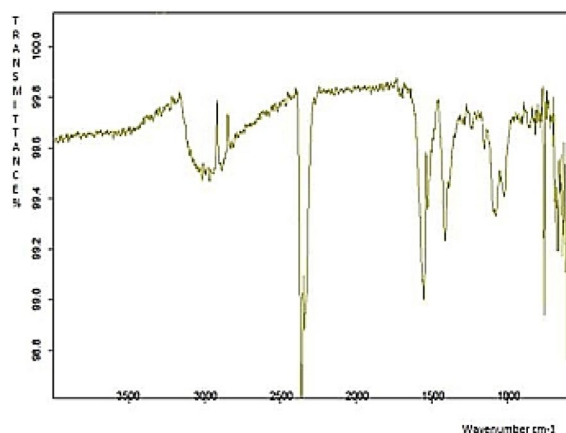


Fig. 5 FT-IR of copper-silver-chitosan nanocomposite

Discussion

Infections caused by opportunistic fungi have been especially important in recent years [32]. *Candida* species are the most common fungi isolated from human infections. *Candida* is a common yeast in opportunistic fungal diseases around the world, which is colonized with abundance on the surface of the skin and mucous membranes [33]. The candidate is the most common cause of invasive fungal infections, which accounts for 70–90% of invasive fungal infections [34].

In recent years, microbial species have shown greater resistance to antimicrobial agents. The limitation of the

Table 1 Results for determination of MIC and MFC values for the studied nanoparticles

Treatment/control	Nanoparticles studied	MIC ($\mu\text{g/ml}$)	MFC ($\mu\text{g/ml}$)	SD ($\mu\text{g/ml}$)
1	Silver nanoparticles	256	260	± 8
2	Copper nanoparticles	385	Not found	± 6
3	Chitosan nanoparticles	> 2560	Not found	± 35
4	Copper/silver nanoparticles	328	352	± 9
5	Copper/chitosan nanoparticles	832	912	± 21
6	Silver/chitosan nanoparticles	438	456	± 11
7	Silver/copper/chitosan nanoparticles	732	482	± 14
8	Silver/copper/chitosan nanocomposite	112	115	± 4
9	5% acetic acid solution (control sample)	> 2560	> 2560	± 44
10	Amphotericin (standard sample)	0.3	1.1	± 0.1



choice of antifungal drugs is one of the important issues in the treatment of fungal diseases [35]. Antifungal drugs that are commonly prescribed have different side effects, such as non-specificity and reduced effects of medications in cancer patients who receive chemotherapy, probiotic therapy or anti-diabetic medications [36, 37]. The use of nanoparticles for the treatment of candidiasis, due to their non-chemical treatment, had less side effects than those commonly used, and we predict that they will be able to effectively treat candidiasis [38, 39]. Copper ions to chitosan nanoparticles are mainly adsorbed by ion exchange resins and surface chelation to adsorb liquid copper nanoparticles. Antibacterial activity of chitosan is affected by a number of factors including bacterial species, concentration, pH, solvent and molecular mass. Due to its excellent antimicrobial properties, chitosan film may be used in food packaging [40].

In a study by Monali Gajbhiye on the effects of silver nanoparticles and their activity on fungi in combination with fluconazole, they concluded that fluconazole in combination with silver nanoparticles showed a more inhibitory effect on *C. albicans* also on *P. glomerata* and *Trichoderma* sp. It also has inhibitory effect. Therefore, the inhibitory effect of the nanoparticles was demonstrated in the presence of fluconazole compound [41]. Kim et al. study also investigated the effects of silver nanoparticles on various dermatophytes and *Candida* species. The inhibitory effects of silver nanoparticles were shown to be even greater than fluconazole and comparable to amphotericin B. The results showed that silver nanoparticles have good activity against mycelia and can have clinical applications [42]. The results of this study indicate that although amphotericin B has an antifungal effect compared to nanoparticles, the properties and effects of nanocomposite are comparable to that of standard materials. In line with previous discussions, El-Rafie et al. [43] also demonstrated the inhibitory effect of silver nanoparticles on fungi.

Panwar [44] showed the inhibitory effect of chitosan nanoparticles on the *C. albicans* biofilm. The results of the study showed that chitosan nanoparticles reduce the metabolic activity of *C. albicans* and interfere with the production of *Candida* yeast biofilms, thus it was introduced as an antifungal agent for the treatment of fungal infections [44]. We have also shown in our studies that the combination of chitosan nanoparticles with silver has been shown to have some antifungal effects. Therefore, the combination of these nanoparticles can be used in the treatment of fungal infections that require further investigation and study of their effects in vivo.

In a study by De Zoysa et al. [45], the antifungal effect of chitosan silver nanocomposite (CAgNC) on *C. albicans* was investigated the overall results showed that CAgNC could negatively affect the physiological and biochemical functions of *C. albicans* and CAgNC as a potential alternative

to antifungal chemotherapy [45]. Yien et al. by investigating the effects of chitosan nanoparticles on fungal microorganisms showed that the inhibitory effect of these nanoparticles is related to the particle size and zeta potential of chitosan nanoparticles and *Aspergillus niger* was inhibited at high concentrations of these nanoparticles, so the nanoparticles concentration alongside the size nanoparticles and zeta potential are involved in its inhibitory effect. They therefore suggested that these nanoparticles could be used as an antifungal agent in the treatment of clinical fungal infections [46]. As the obtained results in our study were clearly showed that copper/silver nanoparticles, copper/chitosan nanoparticles and silver/chitosan nanoparticles individually revealed lower inhibitory and antifungal effects in comparison with silver/copper chitosan nanocomposite.

In a study conducted by Karimiyan and Najafzadeh et al. [47], the effect of four nanoparticles of magnesium oxide, zinc oxide, silicon oxide and copper oxide on *C. albicans* was compared with that of amphotericin B. In this study, the antifungal effects of zinc oxide and copper oxide nanoparticles were confirmed to be used in the future for the treatment of antifungal infections [47]. The most antifungal effect for the nanoparticles studied is related to copper–silver–chitosan nanocomposite and, which has had a significant effect on the growth of *C. albicans* in the laboratory environment compared to other nanoparticles.

Conclusion

The results of this study indicate that although amphotericin B has an antifungal effect compared to nanoparticles, the properties and effects of nanocomposite are comparable to that of standard materials. The results show that this effect was higher for copper–silver–chitosan nanocomposite than for other nanoparticles studied. In this regard, the combination of nanoparticles of chitosan with silver nanoparticles has synergistic effect and has been shown to have some antifungal effects. The most antifungal effect for the nanoparticles studied is related to copper–silver–chitosan nanocomposite and, which has had a significant effect on the growth of *Candida albicans* in the laboratory environment compared to other nanoparticles.

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Author contributions MA: investigate and supervised the findings of this work, wrote the article. MB: designed the study, helped supervise the project, and conceived the original idea. Designed the model and the computational framework and analyzed the data. 3: Supervised the project, contributed to the interpretation of the results. SJH: processed the experimental data, discussed the results and commented on the



manuscript, developed the theoretical framework. AM: provided critical feedback and helped shape the research.

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Compliance with ethical standards

Conflict of interest None to declare.

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