Chitosan nanoparticles as a carrier for *Mentha longifolia* extract: synthesis, characterization and antifungal activity

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A recent study was aimed at determining the antifungal activity of chitosan in both its natural and nanoparticle forms. The present study reports on the preparation and characterization of chitosan nanoparticles and chitosan nanoparticle-incorporated *Mentha longifolia*. The size of the chitosan nanoparticles was 157 nm, and the mint extraction nanoparticles at concentrations of 5%, 10% and 20% were 142, 105 and 63 nm respectively. The surfaces of the chitosan nanoparticles had a positive charge of approximately 33.2 mV, and the mint extract chitosan nanoparticles at 5%, 10% and 20% had charges of approximately 37.5, 36.9 and 36.1 mV respectively. The chitosan nanoparticles had a maximum encapsulation efficiency of 92% at a mint extract concentration of 20%. Mint extract incorporation into chitosan nanoparticles resulted in increased antifungal effects against mycelium growth of *A. niger*.

Keywords: Antifungal activity, characterization, chitosan nanoparticles, *Mentha longifolia*, synthesis.

ANTIMICROBIAL activity in plants happens due to phenolic compounds as well as changes caused in the integrity of the cell membrane and permeability[1]. *Mentha* belonging to the family Lamiaceae, is popularly used in medicine to treat bronchitis, flatulency, anorexia, ulcerative colitis, liver fatigue, larynx irritation, fever, nausea, dyspepsia and stomach ache because of its anti-inflammatory, carminative, antiemetic, diuretic, antispasmodic, tranquilizer, stimulant and emmenagogue properties[2–7]. Mint plants are rich sources of magnesium and iron, which play a great role in human nutrition[8]. Many studies have demonstrated the significant antimicrobial activities of *Mentha* due to the presence of oxygenated monoterpenes in their chemical composition[9–12]. The effect of mint can be attributed to menthol and 1,8-senol, which showed antifungal properties[13]. Studies have also examined the incorporation of plant extract within chitosan nanoparticles. Hence, the aim of this work was to prepare and characterize a combination of chitosan nanoparticles and *Mentha longifolia*, and examine its antifungal efficacy. Chitosan nanoparticles have been used in the delivery of active components because of their ability to provide protection and stability. They provide protection against decomposition or damage due to exposure to oxygen, light, heat, and pressure[14,15], as well as oxidation and enzymatic degradation[16].

Polymers such as chitosan nanoparticles are ideal protectors and can be used for delivery of active compounds[17,18]. Chitosan nanoparticles were loaded into different plants such as carvacrol, oregano, savory, and marjoram[19], sage and savory[20], *Arrabidaea chica*[21], and *Leucas aspera*[22]. The physicochemical characteristics of chitosan nanoparticles such as size, surface area, cationic nature, active functional groups, high permeability towards biological membranes, non-toxicity to humans, cost effectiveness, higher encapsulation efficiency and/or through blending with other components, and broad antifungal activities have led to their increased use in biological applications compared to bulk chitosan, which has not been widely applied as an antifungal agent mainly because of its insolubility in aqueous media and lower antifungal activities[23–26]. Few reports are available on the use of chitosan nanoparticles against plant diseases caused by fungal pathogens[25–27].

Materials and methods

Preparation of chitosan nanoparticles

Chitosan (1%) bought from Sigma-Aldrich (USA) was dissolved in acetic acid and the pH adjusted to 4.6–4.8 with NaOH. It was filtered through a 0.45 membrane (millipore). Sodium tripolyphosphate (TPP) was

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dissolved in deionized water to 1.5% wt/v and then 2 ml of TPP solution was added drop-wise (0.3 ml/min) to 5 ml of chitosan solution with magnetic stirring for 60 min at room temperature, resulting in spontaneous formation of the chitosan nanoparticles[20]. Nanoparticles were purified by centrifugation at 9000 *g* for 30 min to remove the excess unreacted chitosan. Distilled water was used for rinsing chitosan nanoparticles to get rid of any sodium hydroxide.

Preparation of plant extract

Mint leaves (1 g) cut into small pieces were dried, crushed and boiled in 100 ml water and the extract was filtered through a 0.45 mm filter[28].

Preparation of plant extract chitosan nanoparticles

Mint extract had been incorporated into TPP solution prior to the formation of nanoparticles. The mint extract was added drop-wise at three various concentrations (5%, 10% and 20%) with respect to the amount of chitosan and 1% Tween 80 was added in magnetic stirring for 2 h followed by centrifugation. Cellulose membrane (spectra/por dialysis membrane; M_w 3000 Da) was used for removing any free plant extract (non-encapsulated)[21]. The non-entrapped plant extract was recovered from the external aqueous phase after dialysis[21], and was quantified using a calibration curve obtained from extracted solutions of known concentration by measuring the absorbance rate at 485 nm with a spectrometer (Biochrom, Libra S22, UK). The percentage of encapsulation efficiency was defined as:

$$\text{Encapsulation efficiency (\%)} = (\text{total amount} - \text{free amount/total amount}) \times 100.$$

Characterization of nanoparticles

The morphology of chitosan nanoparticles was characterized using a transmission electron microscope (TEM) (JEM-1400, 100 kV) and scanning electron microscope (SEM) (JSM-7610F field emission SEM).

Particle size and zeta potential were determined using a Zetasizer nano series, HT Laser, ZEN 3600 (Malvern Instruments, UK) at a scattering angle of 90° and a temperature of 25°C using samples diluted in different concentrations with de-ionized distilled water. The Fourier transform infrared spectra (Nicolet 6700 spectrometer) at a resolution of 4 cm^{-1} (Thermo Electron Corporation, USA) were used for estimating the structural features of nanoparticles in the range of 400–4000 cm^{-1} using KBr pellets.

Antifungal effect of nanoparticles on Aspergillus niger mycelium growth

The effect of chitosan, chitosan nanoparticles, and mint extract chitosan nanoparticles (20%) on mycelium growth was evaluated on potato dextrose agar (PDA) plates containing different concentrations of nanoparticles, i.e. 0.5, 1 and 2 ml/ml (5 ml mint extract nanoparticles/10 ml PDA, 10 ml mint extract nanoparticles/10 ml PDA and 20 ml mint extract nanoparticles/10 ml PDA). Commercial PDA powder was used (20 g dextrose, 15 g agar and 4 g potato starch as 39 g/1 distilled water) and 5% of additional agar powder added while preparing for higher solidification[29]. The 6 mm diameter fungal mycelium disk was placed at the centre of each petri dish and then was incubated at 25°C. When the mycelia reached the edges of the control plate, the growth of mycelia was determined by measuring the colony diameter expressed as average diameter (mm). Each treatment was repeated three times and the antifungal percentage was calculated as

$$\text{Antifungal (\%)} = T - C/\text{C} \times 100.$$

where C is the linear growth in control plate and T the linear growth after treatment[30].

Results and discussion

Scanning electron microscopy

The surface morphology of the chitosan nanoparticles was determined by SEM (Figure 1). There was a large number of nanoparticles with an almost spherical shape and they were well-separated from one another[29]. SEM images of freeze-dried chitosan nanoparticles prepared by ionic gelation taken. The freeze-dried samples, even with some aggregation of the nanoparticles due to dispersion during freeze-drying, exhibited nanoscale particles with diameters less than 1 mm, which is in accordance with previous studies[31]. Other studies reported an increase in particle size after freeze-drying unmodified chitosan particles[18]. This is a result of aggregation caused by the strong inter- and intra-molecular hydrogen bonding, which could not be avoided even with vortex homogenization[18] which leads to more encapsulation of essential oils[31].

Transmission electron microscopy

Morphological studies of the synthesized nanoparticles were carried out using transmission electron microscopy (TEM). Figure 2 *a* shows that the particles were spherical and had smooth surfaces. This result agrees with that reported by Da Silva *et al.*[20]. TEM images show two layers

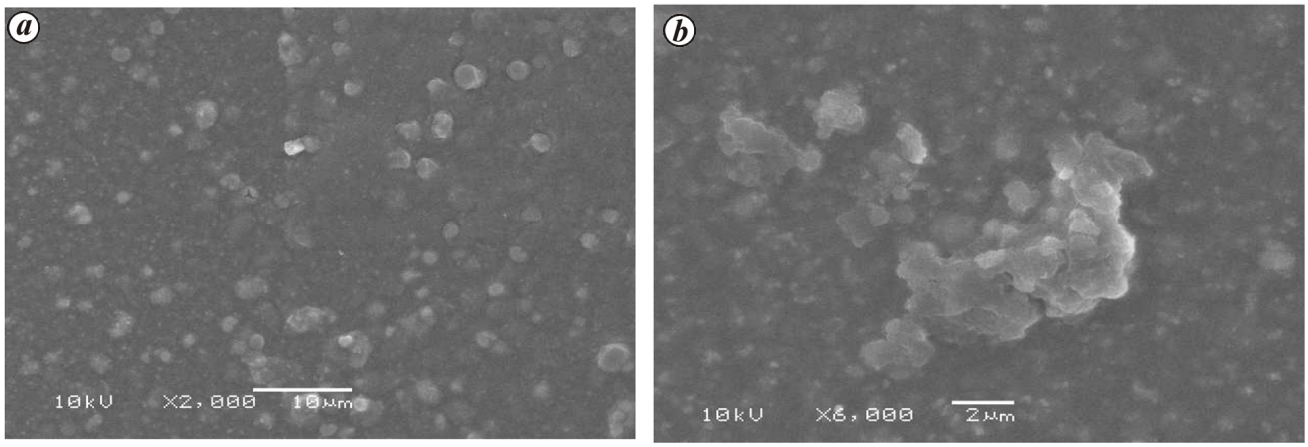

Figure 1. ***a***, SEM image of chitosan nanoparticles freeze dried. ***b***, Incorporated chitosan nanoparticles with mint extract at 20%.

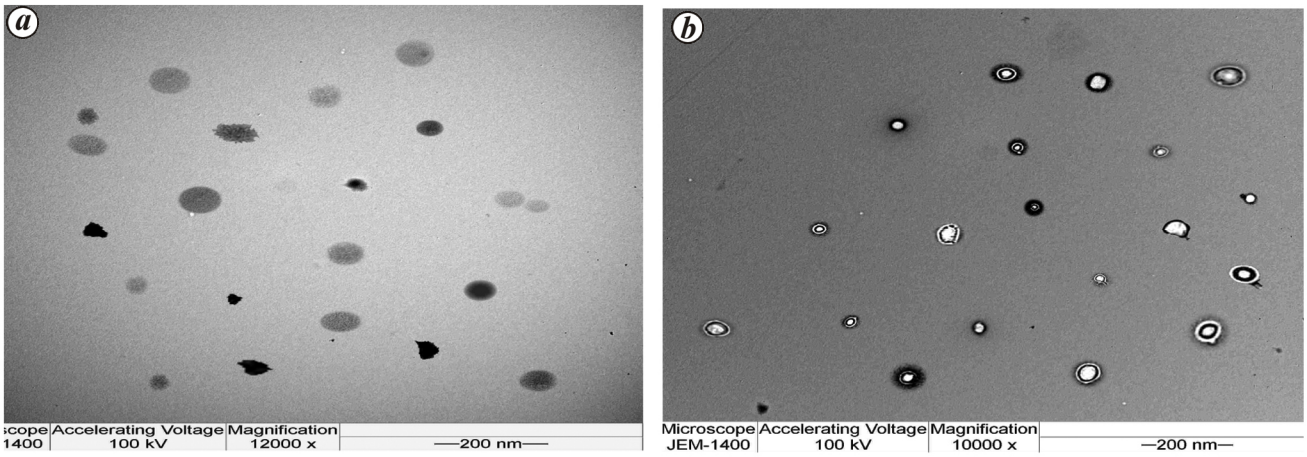

Figure 2. ***a***, TEM image of chitosan nanoparticles. ***b***, Mint extract chitosan nanoparticles.

Table 1. Average particle size and zeta potential values measured using a Nano Zetasizer

Proportion of incorporated mint extract	Size (nm)	Zeta potential (mV)
0% (chitosan nanoparticles without mint extract)	157	+32.5
5%	142	+37.5
10%	105	+36.9
20%	63	+36.1

(outer shell and inner core) in the mint extract chitosan nanoparticles (Figure 2 *b*), which agrees with the one reported by Kulani[16]. The images show that most particles were isolated and only a few were grouped together. This may be due to insufficient time for complete TPP redistribution and particle rearrangement.

Particle size and zeta potential

Interaction between negatively charged tripolyphosphate and positively charge chitosan lead to the preparation of chitosan nanoparticles which was based on ionic gelation[32,33].

The mean size and zeta potential of the nanoparticle suspension were analysed using the Zetasizer analysis as shown in Table 1 and Figure 3. The size of chitosan nanoparticles was 157 nm and mint extraction nanoparticles at 5%, 10% and 20% were 142, 105 and 63 nm respectively. The particle size decreased with increasing mint extract concentration because of the interaction between polymers and extract composition[21,34].

As shown in Figure 3 *b*, the zeta potential results indicated that the surface of chitosan nanoparticles had a positive charge of ~33.2 mV, and the mint extract chitosan nanoparticles at 5%, 10% and 20% exhibited zeta potentials of approximately 37.5, 36.9 and 36.1 mV respectively. The mint extract-chitosan nanoparticles had higher positive value ranges from 36.1 to 37.5 mV compared to chitosan nanoparticles (33.2 mV), which could have been due to an increase in positively charged compounds at the surface of chitosan nanoparticles synthesized with plant extract.

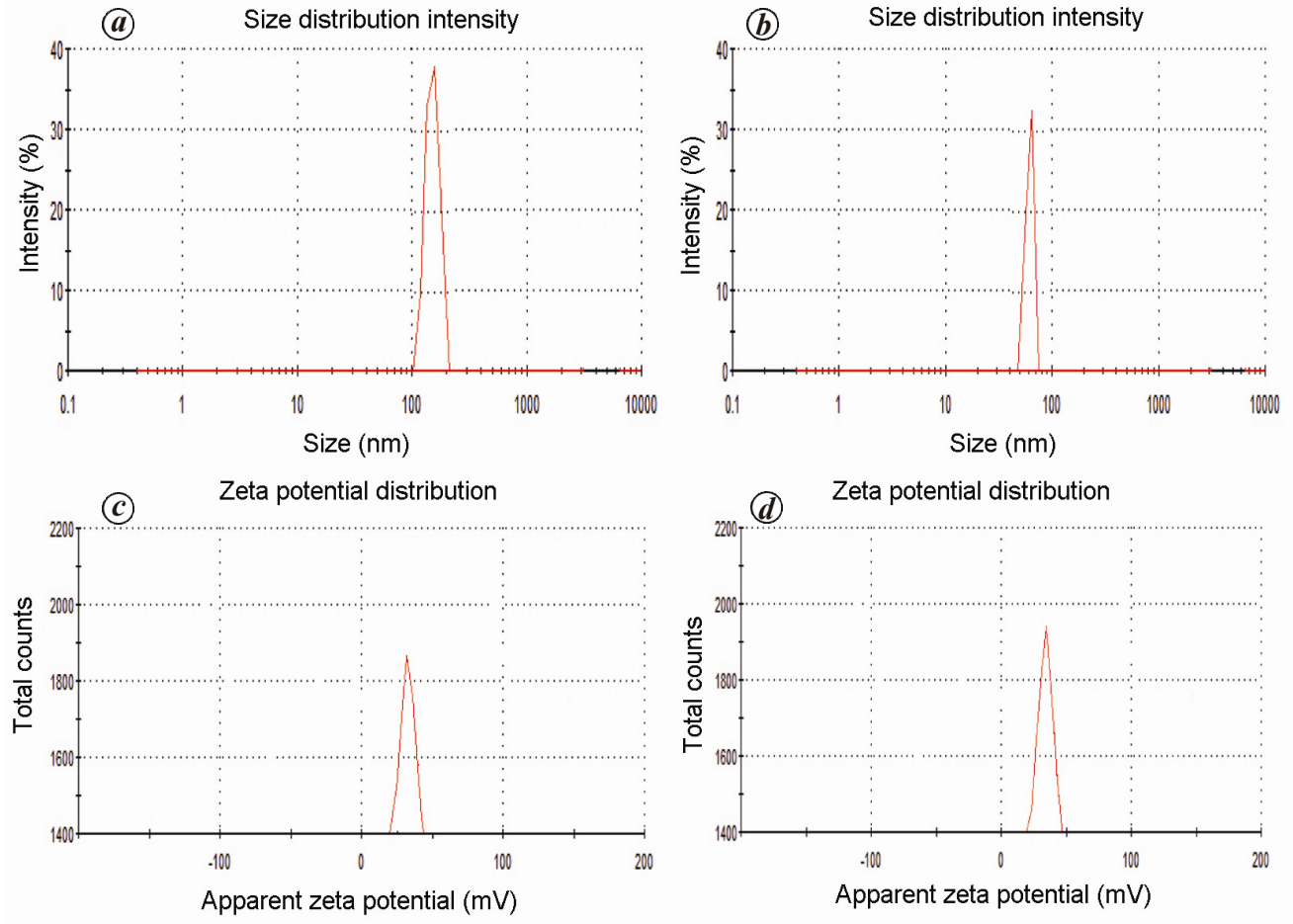

Figure 3. ***a***, Zeta particle size of chitosan nanoparticles. Bar chitosan nanoparticles showed a range of 105–190 nm. ***b***, Zeta particle size of mint-chitosan nanoparticles at 20%. Bar mint chitosan nanoparticles showed a range of 48–75 nm. ***c***, Zeta potential size of chitosan nanoparticles. Bar mint chitosan nanoparticles showed a range of 17–48 mV. ***d***, Zeta potential size of mint-chitosan nanoparticles at 20%. Bar mint chitosan nanoparticles showed a range of 23–48 mV.

The zeta potential is the electrical superficial charge of particles and is influenced by the particle composition and dispersion medium[35]. The electronic dissonance between particles can greatly affect particle stability. As a result, a higher absolute zeta potential value indicates more stability, whereas a lower value indicates colloid instability, which could lead to aggregation[36]. Hence, a zeta potential above ±30 mV has been shown to be stable, as the surface charge prevents aggregation of the particles[35], and this has been documented in similar studies[28,37]. The observed positive zeta potential might be due to the residual amine group, which acts as a carrier of plant extract[38]. SEM, TEM and Zetasizer indicated that the nanoparticle formulation had dispersed without any aggregation[39].

Fourier transform infrared spectra

The Fourier transform infrared (FTIR) spectra of chitosan, chitosan nanoparticles and mint extract nanoparticles are shown in Figure 4. A broad peak of 3263.53 cm^{-1} was attributed to the combination of stretching modes of OH and NH bonds in chitosan[18]. In the sample of chitosan nanoparticles, this band became wider, indicating an enhancement in hydrogen bond due to hydrostatic interaction between amino groups and phosphoric groups in TPP[40].

In addition, the 1650 cm^{-1} peak of the NH_2 bending vibration of chitosan samples shifted to 1628 cm^{-1} for the nanoparticles and the 1153.67 cm^{-1} peak shifted to 1112.84 cm^{-1}. A similar result was observed for chitosan-TPP nanoparticles[41] and the shift attributed to interaction between the amino group and phosphate anion[42].

New absorption bands at 1220.35 cm^{-1}, that appeared in the sample of nanoparticles were due to PO stretching[42,43]. The FTIR of mint extract nanoparticles showed bands at 1300.78, 1600.86 and 1705.85 cm^{-1}, which were absent in chitosan nanoparticles but present in mint extract, indicating successful functionalization/incorporation of the chitosan nanoparticles[42].

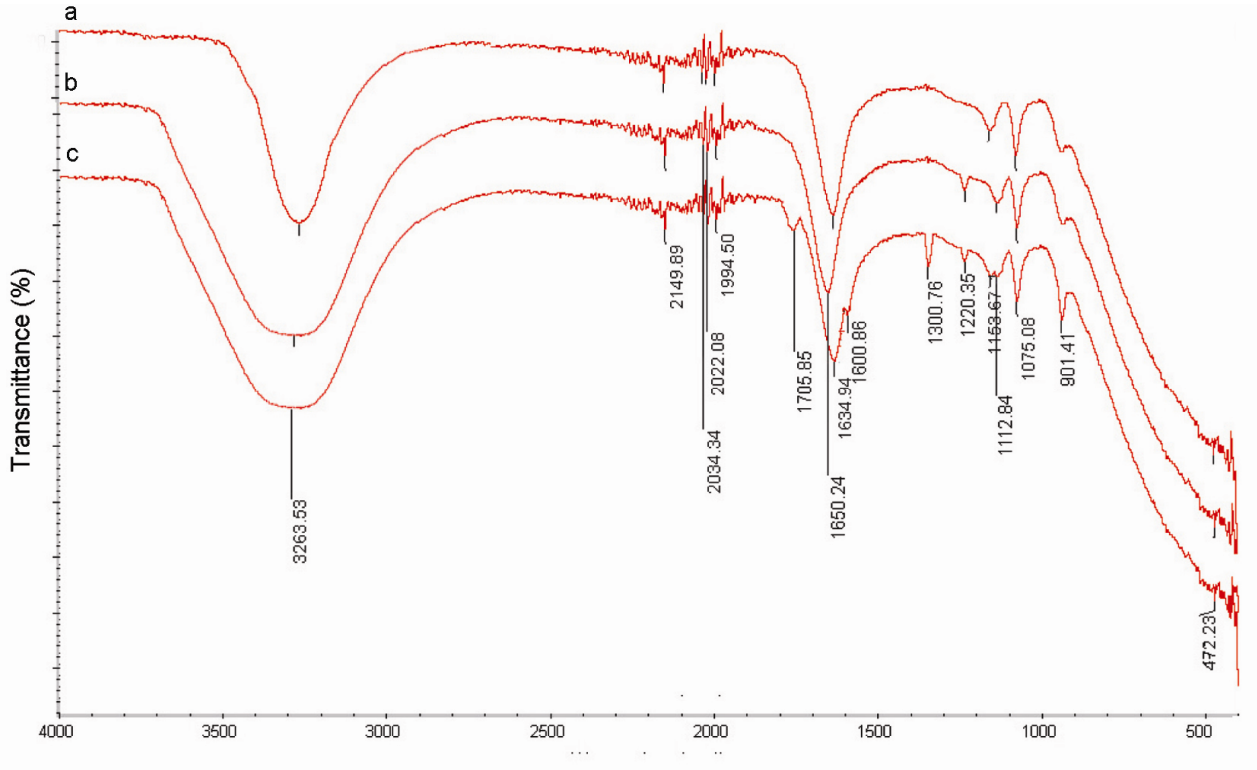

Figure 4. FTIR spectra of chitosan (***a***), chitosan nanoparticles (***b***) and mint extract chitosan nanoparticles (***c***).

Table 2. Loading efficiency (%) of mint extract chitosan nanoparticles

Mint extract concentration (%)	Loading efficiency of mint extract (%)
5	56
10	78
20	92

Encapsulation efficiency

The encapsulation efficiency of nanoparticles was calculated by determining the amount of non-entrapped plant extract in supernatant, measured by a UV-Vis spectrophotometer. The data in Table 2 indicates that the encapsulation efficiency of chitosan nanoparticles was greatly influenced by the increase in plant extract. However, the chitosan nanoparticles had a maximum encapsulation efficiency of 92% when 20% mint extract was incorporated.

Effect of chitosan, chitosan nanoparticles, and mint extract chitosan nanoparticles on mycelium growth

The antifungal effects against *Aspergillus niger* on PDA are shown in Table 3. The data showed clear differences between chitosan, chitosan nanoparticles, and mint extract chitosan nanoparticles (20%) at three different concentrations (0.5, 1 and 2 ml/ml). The inhibitory effect increased as the concentration of chitosan nanoparticles was increased. Loading mint extract in chitosan nanoparticles led to increased antifungal effects compared to chitosan and chitosan nanoparticles. Mint extract chitosan nanoparticles had the greatest inhibitory effect at concentrations of 0.5, 1 and 2 ml/ml (54.4%, 66.7% and 75.6% respectively). Chitosan nanoparticles were the second most effective against mycelia growth of *A. niger*. The growth of mycelia was more obvious on medium amended with chitosan nanoparticles with a positive relationship at three different concentrations. The greatest inhibitory effect was 65.6% at 2 ml/ml, whereas the lowest inhibitory effect was observed on medium with chitosan concentrations of 0.5, 1 and 2 ml/ml (3.3%, 11.1% and 18.8% respectively). Qi *et al.*[43] and Sharma *et al.*[44]. demonstrated that chitosan polymer was less effective as an antifungal agent compared to chitosan nanoparticles due to its characteristics such as small particles with a high surface charge, which enables nanoparticles to adsorb more and provides a higher affinity for binding onto the fungal cell surface. This could be because the negatively charged plasma membrane was the main target site[29,45,46]. Chitosan molecules were located extracellularly whereas chitosan nanoparticles were able to spread intracellularly,

Table 3. Percentage of antifungal activity of chitosan solution, chitosan nanoparticles, and mint extract chitosan nanoparticles against *Aspergillus niger* after 5 days at concentrations of 0.5, 1.0 and 2.0 ml/ml

	0.5 (ml/ml)		1.0 (ml/ml)		2.0 (ml/ml)	
Treatment	Linear growth	Inhibition (%)	Linear growth	Inhibition (%)	Linear growth	Inhibition (%)
Chitosan	8.7	3.3	8.0	11.1	7.3	18.8
Chitosan nanoparticles	6.2	31.1	4.3	52.2	3.1	65.6
Mint extract chitosan nanoparticles	4.1	54.4	3.0	66.7	2.2	75.6
Control	9	0.0	9	0.0	9	0.0

thereby leading to DNA and RNA damage. Additionally, chitosan comprises 10% of the *A. niger* cell wall, which causes more resistance to externally amended chitosan[32,47].

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