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Research article

Formulation and Physicochemical Properties of Submicron Chitosan Dispersion from The Selected Types of Chitosans

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Chitosan, submicron dispersions, physicochemical properties, spore germination

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Abstract. A study was conducted to develop an eco-friendly and sustainable based chitosan using four types of preparation methods, the efficiency of low molecular weight chitosan, medium molecular weight chitosan, high molecular weight chitosan and locally prepared chitosan were investigated. *In vitro* studies showed that low molecular weight chitosan and locally prepared chitosan showed equally best results in inhibiting mycelia growth and spore germination. Further studies were carried out to reduce the viscosity of these two types of chitosan solutions by the formation of submicron chitosan dispersions using sonicator. The physicochemical properties of both submicron dispersions were determined. The findings from this study showed that low molecular weight chitosan requires less energy to produce submicron dispersions.

Şeçilmiş Kitozan Tiplerinden Dağılım Gösteren Submikron Kitozanlarının Formülasyon ve Fizikokimyasal Özellikleri

Anahtar Kelimeler:

Kitozan, submikron dağılım, fizikokimyasal özellikler, spor çimlenmesi

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Özet. Dört farklı hazırlık yöntemi kullanarak kitozana dayalı sürdürebilir ve çevre dostu bir çalışma geliştirilmiştir. Bu yöntemler düşük moleküler ağrlıklı kitozan etkisi, orta moleküler ağrılıklı kitozan, yüksek molekül ağırlıklı kitozan ve yerel kitozandan hazırlanan şekilde incelenmiştir. In vitro bulgular düşük ve orta molekül ağırlıklı olan kitozan ve yerel olarak hazırlananların en iyi misel büyüme ve spor çimlenme durdurucu etki gösterdiği belirlenmiştir. İlerleyen çalışmalarda sonikatör aracılığıyla submikron kitozan oluşturmak suretiyle söz konusu iki kitozanın akışkanlığı azaltılmıştır. Her iki submikron dağılımın fizikokimyasal özellikleri belirlenmiştir. Bulgular, bu çalışmada düşük molekül ağırlığındaki kitozanın submikron dağılım için en az enerji gereksinimi olduğunu göstermiştir.

1. INTRODUCTION

Chitosan is a polysaccharide obtained from the exoskeleton of crustaceans, such as shrimp and crab shells (No and Meyers 1997). Antimicrobial activity of chitosan has been detected towards many bacteria, fungi and yeasts. Several studies showed that the biological activity of chitosan considerably depends on the degree of deacetylation and molecular weight (Ueno *et al*., 1997). These features have a great impact on the solubility of chitosan and its contact with the cell walls of target microorganisms. These factors independently affect the antimicrobial properties of chitosan, though it has been advocated that the effect of molecular weight is greater than the degree of deacetylation (Sekiguchi *et al*., 1994). The application of this polysaccharide is limited due to its low solubility in aqueous dilute acids and the high viscosity of its solutions (Tikhonov *et al*., 2006). Chitosan at concentrations higher than 1.5% showed the limited antimicrobial effect due toits high viscosity (Davis 2011), Therefore it is essential to increase the efficacy of chitosan by reducing its viscosity and surface tension.

Nanotechnology is a new and emerging technique, whereby the viscosity of the solution could be reduced to a desired extent. Furthermore, innovative nanotechnology has been successfully used for the packaging of food (Traver 2006). Submicron dispersion isthe class of emulsions where the droplet size is in the range of 200-1000 nm (Winhab *et al*., 2005). These are widely used in various industries, such as in cosmetics, pharmaceuticals, health care and agrochemicals (Sonneville-Aubrun *et al*., 2004). The small droplet size and high kinetic stability make submicron dispersions suitable for efficient delivery of active ingredients and for penetration through the "rough" texture of the skin (Amselem *et al*., 1998). The formulation of such a submicron carrier of chitosan could result in a slow release of chitosan so that sudden accumulation of chitosan on the fruit surface and in the plant tissues never occurs. No study has been reported on the screening of different molecular weight chitosan against *C. gloeosporioides* of dragon fruit plant, formulation of submicron chitosan dispersions (SCD) and evaluation of physicochemical properties of SCD. Thus, the study was designed to screen different molecular weight chitosans and to evaluate their efficacy against *C. gloeosporioides* of dragon fruit plants, formulation and determination of the physicochemical properties of SCD.

2. MATERIAL AND METHODS

2.1. Materials

Two coriander cultivars (Arslan cultivar improved from *Coriandrum sativum* var. *vulgare* and Gürbüz cultivar improved from *Coriandrum sativum* var. *microcarpum*), registered by the University of Ankara, Faculty of Agriculture, Department of Field Crops on 07 April 2005, were used as plant materials in this study.

Locally prepared crab shell chitosan (Molecular Weight, MW 350 kDa; 95% deacetylated) was purchased from Chitin-Chitosan Research Centre of Universiti Kebangsaan Malaysia. Low molecular weight chitosan from crab shell (MW 50 kDa; 75–85% deacetylated), medium molecular weight chitosan from shrimp shell (MW 400 kDa; 75–85% deacetylated) and high molecular weight chitosan from shrimp shell (MW 760 kDa; ≥ 85% deacetylated) were purchased from Sigma-Aldrich, USA. Brij 56 and Span 20 were used as emulsifiers and were purchased from Merck KGaA, Darmstadt, Germany and Sigma-Aldrich, USA, respectively.

2.2. Isolation of *C. gloeosporioides* and Preparation of Inoculum

Isolation of *C. gloeosporioides* was carried out from infected dragon fruit plants (Zahid *et al*., 2014). Small infected plant with disease lesions were placed in Petri dishes containing potato dextrose agar (PDA, Merck KGaA, Darmstadt, Germany) and incubated at room temperature (28 ± 2°C). In obtaining pure cultures, the colonies were re-isolated after mycelial growth was observed and the isolates were identified on the basis of their morphological and cultural characteristics. The identified cultures of *C. gloeosporioides* were preserved for further use on PDA slants.

2.3. Pathogenicity Test

Pathogenicity test was carried out with some modifications of method developed by Melanie *et al*. (2004), on healthy stems. The isolated pathogen was cultured in potato dextrose broth (PDB) and incubated for 7 days on a rotary shaker at 150 rpm. Mycelium was removed from the harvested conidia

by filtering through four layers of cheesecloth. Sterile purified water was used to adjust the conidial concentration to 1×10^6 conidia ml^{-1}, determined using a hemacytometer. Healthy plant stems were surface disinfected with 1% sodium hypochlorite (NaOCl) for 2 min. A sterile sharp tooth pick was used to make 2 mm deep artificial wounds and each wound was inoculated with 20 µl of conidial suspension using a sterile syringe needle. These stems were laid on moist filter paper in trays wrapped with transparent plastic and incubated at room temperature for 14 days. Symptom development was observed and recorded regularly.

2.4. Preparation of Conventional Chitosan Solution

Chitosan powder (1.0 g) was dissolved in 100 ml purified water containing 0.5 ml (v/v) glacial acetic acid using an overhead stirrer (Model: IKA® RW 14 basic, Fisher Scientific Sdn Bhd., Malaysia). The pH of the solution was adjusted to 5.6 by adding 1N NaOH, using a digital pH meter (Model: Cyber Scan pH 510, Eutech Instruments, Singapore). Four types of chitosan were used for the screening experiment.

2.5. In vitro Antifungal Assay of Conventional Chitosan against *C. gloeosporioides* Isolated from Dragon Fruit Plants

2.5.1. Inhibition of radial mycelial growth

The *in vitro* antifungal activity of conventional chitosan was determined by placing a 5 mm diameter disc from the pure culture of *C. gloeosporioides* in the centre of PDA dish containing chitosan solution at 0.5, 1.0, 1.5 and 2.0%. Petri dishes containing only PDA were used as controls. All the Petri dishes were incubated at room temperature (28 ± 2°C) until the radial mycelial growth of the control dishes reached the edge of the dish. The percentage inhibition in radial mycelial growth (PIRG) was calculated using the formula described by Al-Hetar *et al*. (2010).

$$\text{PIRG} = \frac{R_1 - R_2}{R_1} \times 100$$

where; R_1 stands for mycelial growth in control plates and R_2 stands for mycelial growth in treated plates.

2.5.2. Conidial germination inhibition test

The conidial germination inhibition test was carried out using the cavity slide technique (Zahid *et al*., 2012).The number of germinated conidia was counted in 10 microscopic fields in 20 replicated dishes and presented as percent inhibition. The percentage inhibition in germination was calculated by the method of Cronin *et al*. (1996).

2.5.3. Dry weight of mycelium

Potato dextrose broth (PDB) and chitosan solutions were separately autoclaved at 121 °C for 20 min and 100 ml of chitosan was added to the PDB (100 ml) in 250 ml Erlenmeyer flasks. Three mycelial discs (5 mm) from a 10 days old culture were added to each flask and incubated at room temperature for 14 days. Pre-weighed Whatman no. 1 filter paper was used to filter mycelium and dried in an oven at 100 °C for 24 h, and then weighed. The dry weight of mycelium was calculated by the following formula (Zahid *et al*., 2012).

$$\text{Dry weight of mycelium} = \frac{\text{Biomass of control sample-Biomass of treated sample}}{\text{Biomass of control sample}}$$

2.5.4. Viability of spores

Viability of spores was measured using potato dextrose broth (PDB) containing chitosan as growth media. A spore suspension of *C. gloeosporioides,* adjusted to 200 spores ml^{-1} was prepared using 10 days old culture plates incorporated with different concentrations of conventional chitosan along with submicron chitosan dispersions of different droplet sizes. An aliquot of 1 ml of the suspension was added to PDA dishes and incubated at room temperature for 4 days. After incubation, the spore population density in each treatment was measured as a percent of sporulation using the following formula (Al-Hetar *et al*., 2010).

$$\% \text{ Sporulation} = \frac{\text{Number of spores in treated sample}}{\text{Number of spores in control sample}} \times 100$$

2.6. Methods for The Preparation of Submicron Chitosan Dispersions

Submicron chitosan dispersions were prepared using a new nano emulsifying alcohol free system (Zahid *et al*., 2012). Chitosan was dissolved separately in 0.5% (v/v) glacial acetic acid (Sigma-Aldrich, USA) and was used as an oil phase. The emulsifiers, Brij 56 (polyethylene glycol hexadecyl ether) and Span 20 (sorbitan monolaurate), were mixed with each other in a ratio of 1:1. The chitosan solution and emulsifier mixture (4:1) were mixed together and subjected to ultrasonication in an ultrasound water bath (Model: Bandelin Sonorex, Germany) at 25 °C. Subjecting to ultrasound for different time periods, submicron chitosan dispersions with droplet sizes of 200, 400, 600, 800

and 1000 nm were obtained. The droplet size was measured using a dynamic light scattering (DLS) technique (Zetasizer NanoZS, Malvern, UK); utilising an argon laser (λ=633 nm) at a scattering angle of 173°.

2.7. Physicochemical Analysis of Submicron Chitosan Dispersions

2.7.1. Zeta potential and stability determination

The zeta potential values of SCD were measured using a Zetasizer (Freitas and Müller 1998). The stability of SCD was determined by measuring the size of the droplets in nm at 25 °C and was expressed in days.

2.7.2. Measurement of viscosity

Viscosity of SCD was measured using a Brookfield Viscometer (Brookfield LVDV-I Prime, Brookfield Engineering Laboratory Inc., USA) equipped with different spindles. The operating principle of the viscometer is to drive the spindle through the calibrated spring immersed in the testing liquid. By using the spindle No. 62 and 250 ml of the liquid in a glass beaker the viscosity was measured and the data was expressed in centipoises (cp) within a range that a torque is required to rotate the spindle for 10% or more by reacting at 100 rpm for 1 minute.

2.7.3. Determination of pH

The pH of the submicron chitosan dispersions was determined using a pH meter (Model: Cyber Scan pH 510, Eutech Instruments, Singapore).

2.7.4. Release of chitosan from submicron dispersions

The release of chitosan from SCD was measured using the method described by Nitschke *et al.* (2011) with slight modifications. Briefly, samples (3 ml) were dissolved in 100 ml of 1M NaOH solution. The mixture was boiled for two hours at 100 °C in a water bath with continuous agitation. The mixture was filtered and the residues collected were dissolved in 20 ml of 10% acetic acid. The mixture was again boiled for one hour in a water bath. This mixture served as the standard solution for chitosan determination. A aliquot of 2 µl sample of the standard solution was dyed with 1 µl of 1.0% lugol's solution and the absorbance was determined at 450 nm using a UV-Vis spectrophotometer (Varioskan Flash Multimode Reader, Thermo Fisher Scientific, USA) for 3 min. A standard curve was prepared using 0.5 to 5 ml of chitosan solution.

2.8. Statistical Analyses

A completely randomised design (CRD) with factorial combination was used for all the experiments and the data were subjected to analysis of variance (ANOVA) using statistical analysis system SAS® version 9.1. Duncan's multiple range test (DMRT) was used for comparing treatment means and statistical significance was assessed at $P<0.05$. Four replicates of twenty units for each treatment were used in all the parameters tested and all the experiments were repeated twice. The differences between the repeated measurements were tested using the UNIVARIATE procedure in SAS®. Variations among different sets of experiments were negligible and the treatment effect was similar, so the data was pooled before analysis.

3. RESULTS AND DISCUSSION

3.1. Isolation and Identification of *C. gloeosporioides* from Dragon Fruit Plants

This pathogenic fungus was identified as *C. gloeosporioides* (Penz.&Sacc.) The fungus was isolated from anthracnose lesions of the dragon fruit plants. The isolates grown on PDA medium at 28 ± 2 °C for 10 days were identified on the basis of previous studies (Masyahit *et al.*, 2009) (Fig. 1a). The fungus had whitish-orange colonies (Fig. 1b), with acervuli having conidiogenous cells bearing capsule-shaped conidia (Fig. 1c). Its morphology was the same as that described previously by Masyahit *et al.* (2009). This demonstrated that *C. gloeosporioides* is the causal agent of anthracnose in dragon fruit plants and is the most common fungus causing latent infections in dragon fruit during postharvest storage.

3.2. Pathogenicity Test

The anthracnose symptoms produced on inoculated stems were similar to those found in natural field conditions. Typical symptoms appeared on stems after 3 days of inoculation (Fig. 2a) and the inoculated stem showed severe rotting after 10 days (Fig. 2b). Similar results were reported by Masyahit *et al.* (2009) where they observed necrotic lesions with yellowish brown centres which later coalesced to an extensive rot. *C. gloeosporioides* was also found to be the causal organism of anthracnose in white fleshed dragon fruit plants in Florida, USA (Palmateer *et al.*, 2007) and in Taiwan (Taba *et al.*, 2006). In Brazil, *C. gloeosporioides* was found to be the causal organism

of anthracnose in yellow genotypes of dragon fruit plants (Takahashi *et al*., 2008).

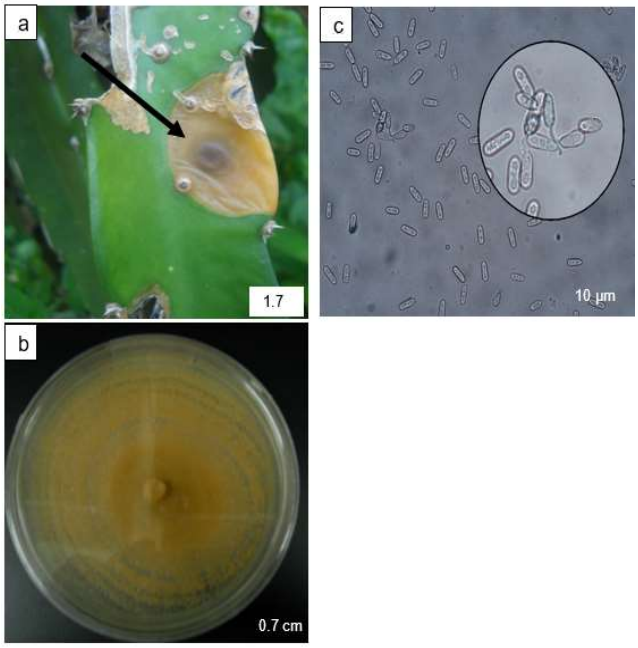

Figure 1. Symptoms of anthracnose on dragon fruit: a)Arrow showing anthracnose lesion on shoot, b)Pure culture of *C. gloeosporioides* and c)Conidia.
Şekil 1. Kaktüs meyvesinde *antraknoz semptomu: a)ok antroknoz lezyonunu göstermektedir, b)Saflaştırılmış C. gloeosporioides* ve c)Conidia kültürleri.

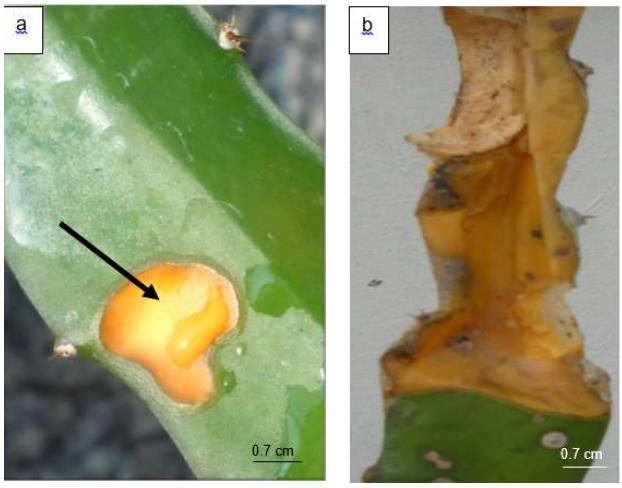

Figure 2. Pathogenicity test showing lesion on shoot after a)3 days and b)10 days of inoculation.
Şekil 2. Gövdedeki patojenite testinin a)3 gün ve b)10 günlük kültür sonundaki gösterimi.

3.3. Effect of Chitosan on Radial Mycelial Growth of *C. gloeosporioides*

Inhibition of radial mycelial growth of *C. gloeosporioides* was observed after 10 days of incubation at 28 ± 2 °C using different types of chitosan (locally prepared, low molecular weight, medium molecular weight and high molecular weight). The growth decreased significantly ($P < 0.05$) by the different types of chitosan at all concentrations tested as compared to the control (Fig. 3). The maximum percentage inhibition (almost 92% and 93%) in growth was observed with 2.0% low molecular weight chitosan and 2.0% locally prepared chitosan, respectively. Medium and high molecular weight chitosans also inhibited the radial mycelial growth of *C. gloeosporioides* but with lower efficacies.

Similar results were obtained when chitosan was used to inhibit the radial mycelial growth of *Rhizopus stolonifer* (El Ghaouth *et al*., 1992) and various fungal species (Hirano and Nagao, 1989). It is believed that the antifungal activity of chitosan varies with the degree of deacetylation, molecular weight, concentration and pH of the solvents (Sekiguchi *et al*., 1994; Guo *et al*., 2006; Junang *et al*., 2009). In a recent study by Hernández-Lauzardo *et al.* (2008), the inhibition of mycelial growth of *R. stolonifer* was more in low molecular weight chitosan as compared with high molecular weight chitosan, while the high molecular weight chitosan affected sporulation more than low and medium molecular weight chitosan. In an earlier study, it had shown that a lower level of deacetylation would enhance the antifungal property of chitosan (Stössel and Leuba 1984). In addition, the intensity of action of chitosan on fungal cell walls depends on the concentration and pH of the chitosan solution (Stössel and Leuba 1984).

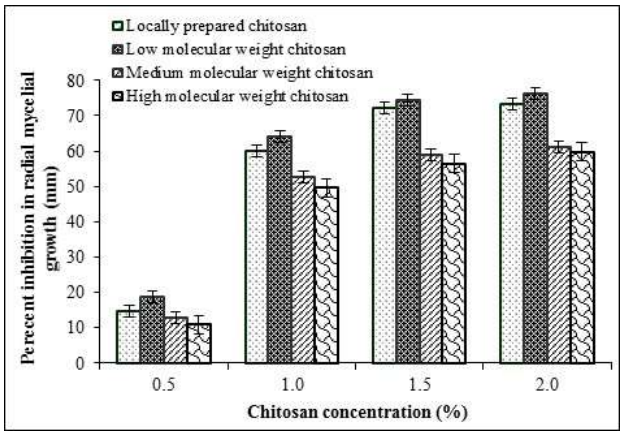

Figure 3. Effect of concentrations and types of chitosan on radial mycelial growth of *C. Gloeosporioides* after 10 days of incubation. The vertical bars represent the standard error of means for four replicates.
Şekil 3. Kitozanın konsantrasyonu ve tipinin C. Gloeosporioides'nın radyal misel büyümesine olan 10 günlük kültür sürecinde etkisi. Barlar dört tekrarlı standart hatayı göstermektedir.

3.4. Effect of Chitosan on Conidial Germination of *C. gloeosporioides*

The germination of *C. gloeosporioides* conidia was significantly (P<0.05) inhibited by all types of chitosan tested at all concentrations as compared to the control (Fig. 4). The maximum percentage inhibition in conidial germination (almost 89 and 87%) was observed with 2.0% low molecular weight chitosan and 2.0% locally prepared chitosan, respectively Medium and high molecular weight chitosans also inhibited the conidial germination of *C. gloeosporioides* but with lower efficacies. Chitosan concentrations are directly proportional to the conidial germination inhibition (Al-Hetar *et al*., 2010). Studies conducted on cultures of *Rhizoctonia solani* revealed that the percentage germination of fungal spores decreased with increased concentration of chitosan in the medium and also with the degree of deacetylation. Zhang *et al.* (2003) reported that 80% inhibition of conidial germination for *Phomopsis asparagi* and 95% for *Fusarium oxysporum, Rhizoctonia solani* and *Cucumerinum owen*when treated with the decrease in molecular weight and degree of deacetylation of chitosan.The degree of deacetylation is a basic factor in solubility and charge development of chitosan, where the dominating reactive sites are NH_2 and OH groups. Hence, as the degree of deacetylation is reduced, more free amino groups present in chitosan which could lead to higher antimicrobial activity (Andres *et al*., 2007). The antifungal mechanism of chitosan involves cell wall morphogenesis with chitosan molecules interfering directly with fungal growth (El Ghaouth *et al*., 1992).

3.5. Effect of Different Types of Chitosan on Dry Weight of Mycelium and Spore Viability of *C. gloeosporioides*

The dry weight of mycelium of *C. gloeosporioides* was significantly (P< 0.05) reduced by all types and concentrations of chitosan as compared to the control (Fig. 5). The minimum dry weight of mycelium was observed at 0.94g and 0.98g in Petri dishes treated with 2.0% low molecular weight chitosan and 2.0% locally prepared chitosan respectively. In contrast, medium and high molecular weight chitosans resulted in less reduction of mycelium dry weight.

The spore viability of *C. gloeosporioides* was significantly (P<0.05) reduced by all types and concentrations of chitosan as compared to the control (Fig. 6). The highest loss in spore viability (almost 20 and 21%) was observed in Petri dishes treated with 2.0% low molecular weight chitosan and 1.5% low molecular weight chitosan, respectively. Medium and high molecular weight chitosans also showed inhibition in spore viability but with lower efficacies.

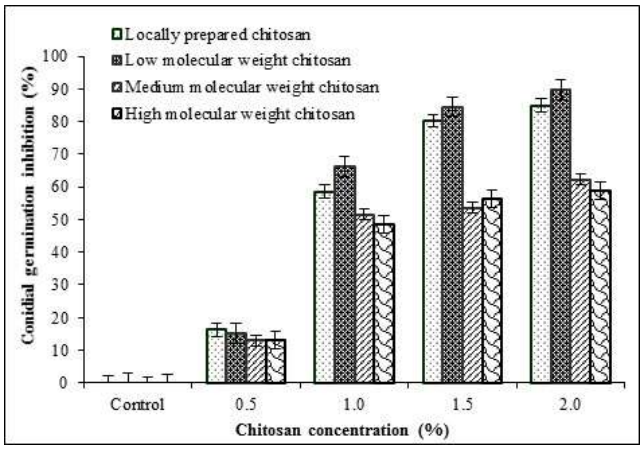

Figure 4. Effect of concentrations and types of chitosan on conidial germination inhibition (%) of *C. gloeosporioides*. The vertical bars represent the standard error of means for four replicates.

Şekil 4. Kitozanın konsantrasyonu ve tipinin C. Gloeosporioides'nın çimlenme durdurmasına olan 10 günlük kültür sürecindeki etkisi. Barlar dört tekrarlı standart hatayı göstermektedir.

Chitosan affected the dry weight of the mycelium and sporulation, demonstrating its effects on various developmental stages of *C. gloeosporioides*. As discussed earlier, molecular weight and degree of deacetylation strongly affect antimicrobial activity of chitosan, with varied effect based on the growth stage of the microorganism (Andrew, 2001). Generally, the viability of spores has been reduced effectively by chitosan (El Ghaouth *et al*., 1992; Sashai and Manocha 1993). Moreover, chitosan has the potential to induce regulatory changes in fungi, but it is presumed to be fungistatic rather than fungicidal (El Ghaouth *et al*., 1992; Raafat *et al*., 2008). After an initial screening experiment, two types of chitosan (low molecular weight and locally prepared) were selected to prepare submicron dispersions and for further *in vitro* evaluation against *C. gloeosporioides* isolated from dragon fruit plants.

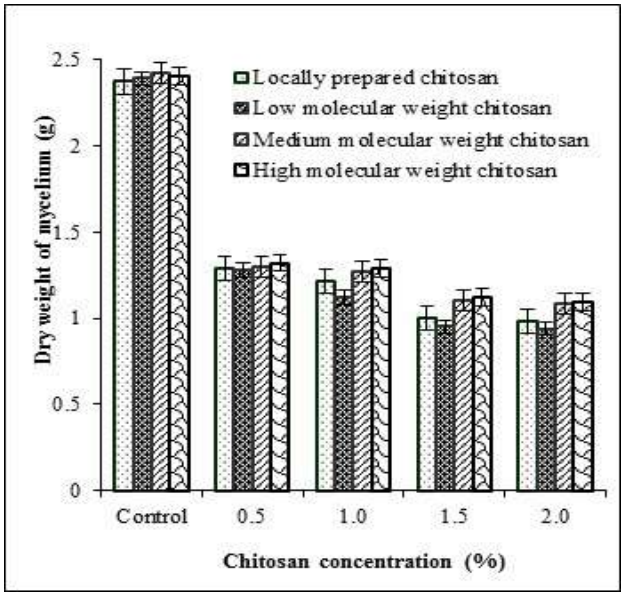

Figure 5. Effect of concentrations and types of chitosan on dry weight of *C. gloeosporioides* mycelium (g). The vertical bars represent the standard error of means for four replicates.
Şekil 5. Kitozanın konsantrasyonu ve tipinin C. Gloeosporioides misel kuru ağılırğına (g) etkisi. Barlar dört tekrarlı standart hatayı göstermektedir.

3.6. Formulation of Submicron Chitosan Dispersions

A longer period of sonication was applied to obtain smaller droplest for low molecular weight chitosan. Similarly, with an increase in chitosan concentration, a longer time was required for sonication (Table 1). In contrast, less time of sonication is required to achieve larger droplets for low concentration of chitosan. Locally prepared chitosan required a little more time for sonication as compared to low molecular weight chitosan for all concentrations and droplet sizes.

The result regarding the decrease droplet size of submicron chitosan dispersions was increased by the sonication time and the energy input into the system (Jafari *et al.,* 2007). It has previously been reported that the time of sonication required to prepare submicron dispersions varies with the type and concentration of the materials (Karbstein and Schubert 1995; Lobo and Svereika 2003).

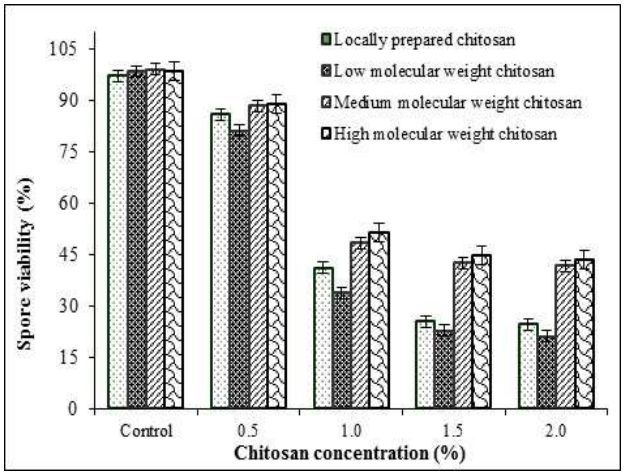

Figure 6. Effect of concentrations and types of chitosan on spore viability (%) of *C. gloeosporioides*. The vertical bars represent the standard error of means for four replicates.
Şekil 6. Kitozanın konsantrasyonu ve tipinin C. Gloeosporioides spor canlılığına (%) olan etkisi. Barlar dört tekrarlı standart hatayı göstermektedir.

Table 1. Time of sonication (min) to obtain different droplet sizes at various concentrations of chitosan.
Çizelge 1. Çeşitli konsantrasyonlarda farklı boyutlarda kitozan damlaları elde etmede sonikasyon süresi.

Type of chitosan	Chitosan concentration (%)	Droplet size (nm)				
		200	400	600	800	1000
Time of sonication (min)						
Low molecular weight	**0.5**	20.4 g	16.9 ij	13.6 k	11.0 m	8.4 n
	1.0	25.9 de	23.2 f	18.9 h	16.4 j	12.2 l
	1.5	30.1 bc	25.6 de	22.1 fg	18.1 h	15.5 k
	2.0	35.2 a	30.4 bc	24.5 e	20.2 g	18.5 h
Locally prepared	**0.5**	21.3 g	17.2 i	14.7 jk	12.4 l	13.3 k
	1.0	27.8 de	25.7 de	22.0 fg	17.8 i	16.0 j
	1.5	32.5 b	28.7 c	25.2 de	21.1 g	18.4 h
	2.0	36.4 a	32.1 b	28.5 c	23.0 f	21.4 g

Means with different letters are significantly different using DMRT test (P< 0.05).

3.7. Physicochemical Properties of Submicron Chitosan Dispersions

3.7.1. Zeta potential

With an increase in the concentration of chitosan tested, the value of zeta potential also increased significantly ($P<0.05$) (Table 2). The zeta potential was highly dependent on the energy input in terms of time of sonication, however, the values for low molecular weight chitosan droplets were higher than those for locally prepared chitosan. The zeta potential value decreased with an increase in droplet size for both of the chitosans tested. The values also increased with the increase in sonication time. The zeta potential value of 200 nm droplets of 0.5% chitosan was lower than the same droplet size of 2.0% chitosan. The larger droplet sizes had lower zeta potential values due to low energy input into the system. Droplets with 1000 nm size of 2 % chitosan had higher zeta potential as compared to droplets with 200 nm size of 0.5% chitosan. The locally prepared chitosans showed slightly lower values of zeta potential. The difference in zeta potential values for low molecular weight chitosan and locally prepared chitosan were probably due to the effect of molecular weight (Rinaudo *et al*., 1999; Rusu-Balaita *et al*., 2003).

Zeta potential is a parameter that characterizes the electrochemical equilibrium on interfaces (McNaught and Wilkinson 1997), which depends on the properties of the liquid and surfactant. Electrostatic repulsion between droplets increases with an increase in the value of zeta potential (Lagaly 1984). In the present study, the increase in zeta potential with the decrease in droplet size could be ascribed to increased energy input from sonication (Riddick 1968).

3.7.2. Stability

The stability of dispersions decreased with increase in droplet size for both of the chitosans tested (Table 3) and also depended on the concentration of the chitosan. Stability of the low molecular weight submicron chitosan dispersions was higher than that of the locally prepared chitosan, i.e. 6.0 and 5.0 days for 200 nm droplets of 0.5% low molecular weight and locally prepared chitosan, respectively. However, stability increased with increase in concentration.

Stability of the submicron dispersions was directly proportional to the zeta potential value (Fig. 7). The equation shows that each unit increase in zeta potential resulted in 2.35 units increase in stability of low molecular weight chitosan (Fig. 7a). For locally prepared chitosan, every unit increase in zeta potential resulted in 1.58 units increase in stability (Fig. 7b). This is in agreement with the previous findings of Riddick (1968), where a reduction in physical stability was observed with a reduction in zeta potential. It reflects the electrical potential of submicron dispersions is influenced by the dispersion medium and its composition. Dispersions with high zeta potential retain their stability in suspension, as the similar charges on the surface of dispersions prevent accumulation of the particles (Rajendran *et al*., 2010).

The difference in stability of different types of submicron chitosan dispersions mainly depends on the degree of deacetylation. Chitosan with less deacetylation possesses more positive charges on its surface, such that the similar charges resist the formation of aggregates (Jansson 2010).

Table 2. Zeta potential (mV) of different droplet sizes and various concentrations of chitosan.
Çizelge 2. Farklı damla boyutlarının Zeta potansiyeli (mV) ve kitozan konsantrasyonları.

Type of chitosan	Chitosan concentration (%)	Droplet size (nm)				
		200	400	600	800	1000
Zeta potential (mV)						
Low molecular weight	**0.5**	20.1 e	17.0 i	15.2 l	15.9 k	14.8 m
	1.0	21.3 d	19.1 ef	17.5 h	15.3 kl	16.6 j
	1.5	23.2 bc	22.3 c	22.7 c	21.1 d	18.2 gh
	2.0	25.5 a	24.4 b	24.1 b	21.2 d	22.1 c
Locally prepared	**0.5**	16.7 j	14.3 m	13.9 n	12.2 o	12.6 no
	1.0	17.1 i	15.3 l	14.9 m	15.8 k	14.1 m
	1.5	19.3 f	18.8 g	16.2 j	16.5 j	16.2 j
	2.0	21.2 d	20.7 d	18.8 g	17.4 i	18.5 gh

Means with different letters are significantly different using DMRT test ($P< 0.05$).

Table 3. Stability (days) of droplet sizes at various concentrations of chitosan.
Çizelge 3. Değişken kitozan konsantrasyonlarında damla boyutlarının stabilitesi(günler).

Type of chitosan	Chitosan concentration (%)	Droplet size (nm)				
		200	400	600	800	1000
Stability (days)						
Low molecular weight	**0.5**	6.0 b	5.0 c	5.0 c	5.0 c	4.0 d
	1.0	6.0 b	6.0 b	5.0 c	5.0 c	4.0 d
	1.5	7.0 a	6.0 b	6.0 b	6.0 b	5.0 c
	2.0	7.0 a	7.0 a	7.0 a	6.0 b	6.0 b
Locally prepared	**0.5**	5.0 c	5.0 c	4.0 d	4.0 d	4.0 d
	1.0	5.0 c	5.0 c	4.0 d	4.0 d	4.0 d
	1.5	6.0 b	5.0 c	5.0 c	5.0 c	5.0 c
	2.0	6.0 b	6.0 b	5.0 c	5.0 c	5.0 c

Means with different letters are significantly different using DMRT test (P< 0.05).

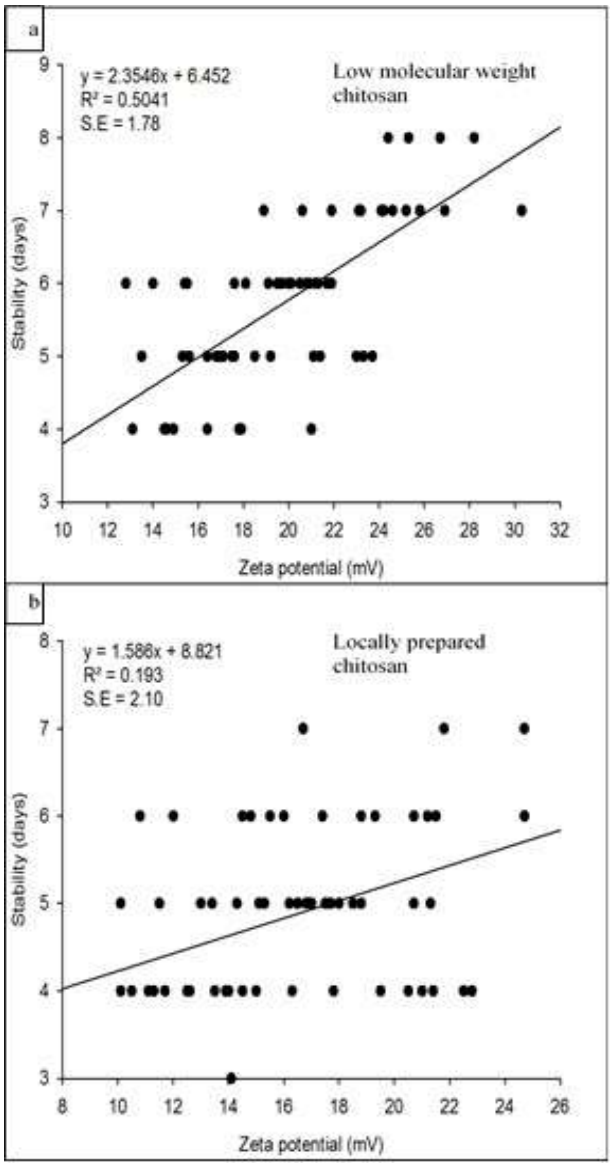

Figure 7. Relationship between zeta potential and stability of submicron chitosan dispersions of a)low molecular weight chitosan and b)locally prepared chitosan at P<0.05.
Şekil 7. Zeta potansiyeli ve submikton kitozan dağılımı a)düşük molekül ağırlıklı kitozan ve b)yerel hazırlanan kitozan arasındaki ilişki.

3.7.3. Viscosity and pH

The viscosity of the dispersions also increased with increase in the concentration of chitosan and in droplet size for both of the chitosans tested (Table 4). The solutions prepared with locally prepared chitosan had higher viscosity than the low molecular weight chitosan solutions. The increase in viscosity with the increase in droplet size and chitosan concentration could be ascribed to the greater time of sonication and therefore the greater energy input required to obtain smaller submicron dispersion droplets. Increased sonication time leads to higher temperature of the submicron dispersion, thus reducing the viscosity of the solution. The temperature was monitored using a thermometer and constantly maintained at 25 °C by adding water in the sonicator as it is believed temperature is the most important factor which reduces the viscosity and interfacial tension of dispersions (McClements 2005). Schuhmann (1995) also reported that higher temperature caused the destabilisation of droplets and decreased the viscosity.

3.7.4. Release of chitosan from submicron dispersions

The release of chitosan from submicron dispersions decreased with increase in the concentration of chitosan and in the droplet size for both of the types of chitosan tested (Table 5). The solutions prepared with locally prepared chitosan showed faster release of chitosan as compared to the low molecular weight chitosan solutions. The results of this study showed that the release of chitosan is related to the chitosan concentration, droplet size and molecular weight of chitosan (Hejazi and Amiji 2003). The release of chitosan from submicron dispersion at lower concentrations was faster. This could be attributed to the network of

emulsifier and chitosan at lower concentration containing a large volume of free moving liquid molecules, which results in quick release of active ingredient from the emulsifiers (Desai and Park 2005). The molecular weight of chitosan also affects the release of chitosan from the matrix of emulsifier. Chitosan of higher molecular weight has higher chain relaxation ability which results in an increase in the polymer chain entanglement of chitosan and emulsifiers (Agnihotri *et al.*, 2004). Therefore, variation in the molecular weight of chitosan results in different release profiles of dispersions.

Table 4. Viscosity (cp) of different droplet sizes and various concentrations of chitosan.
Çizelge 4. Farklı damla boyutlarının akışkanlığı (cp) ve çeşitli kitozan yoğunlukları.

Type of chitosan	Chitosan concentration (%)	Droplet size (nm)				
		200	400	600	800	1000
Viscosity (cp)						
Low molecular weight	**0.5**	12.5 w	19.6 w	24.4 v	36.3 u	42.2 tu
	1.0	17.6 w	22.8 v	48.4 t	69.1 s	75.1 r
	1.5	204.4 o	323.4 n	428.6 m	546.2 k	728.3 g
	2.0	541.5 k	582.7 j	756.3 g	1092.5 e	1462.4 c
Locally prepared	**0.5**	38.2 u	53.1 t	64.2 s	92.9 qr	119.1 q
	1.0	49.1 t	72.8 r	89.4 r	118.4 q	156.5 p
	1.5	324.6 n	510.9 kl	614.5 i	782.7 g	1096.6 e
	2.0	645.5 h	894.7 f	1292.2 d	1564.1 b	1783.7 a

Means with different letters are significantly different using DMRT test (P< 0.05).

Table 5. Release of chitosan (mg ml^{-1}) from submicron chitosan dispersions at various droplet sizes and chitosan concentrations.
Çizelge 5. Farklı damla boyutlarındaki submikron kitozan dağılımıdan salınan kitozan ve konsatrasyonları.

Type of chitosan	Chitosan concentration (%)	Droplet size (nm)				
		200	400	600	800	1000
Chitosan release (mg ml^{-1})						
Low molecular weight	**0.5**	2.11 ab	2.07 ab	1.54 d	1.28 ef	1.08 hij
	1.0	1.12 gh	1.10 ghi	1.22 ef	1.03 g-k	0.96 jkl
	1.5	0.65 jkl	0.71 lm	0.81 klm	0.78 lm	0.64 lm
	2.0	0.55 m	0.47 m	0.49 m	0.57 m	0.55 m
Locally prepared	**0.5**	2.32 a	2.18 a	2.31 a	1.84 bc	1.82 bc
	1.0	1.26 ef	1.29 ab	1.28 ef	1.36 e	1.18 fg
	1.5	1.14 gh	1.16 g	1.07 ghi	1.03 g-k	1.04 g-k
	2.0	1.04 g-k	1.06 ghi	0.98 jkl	0.68 klm	0.69 lm

Means with different letters are significantly different using DMRT test (P< 0.05).

4. CONCLUSION

This study showed that the antimicrobial properties of chitosan depend on the degree of deacetylation and molecular weight of the chitosan. Chitosan with low molecular weight showed better antifungal activity than chitosan with medium or high molecular weight. Similar to the antifungal effects, the properties of submicron chitosan dispersions were also highly dependent on the molecular weight of chitosan. Further studies are required to investigate the mechanism behind the antifungal effects of submicron chitosan dispersions in detail.

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