Prevention of Deteriorative Damage of Post-Harvest Guava by the antimicrobial effect of a Combination of Low-Molecular-Weight (LMW) Chitosan and Nano-silicon Dioxide

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Abstract

Guava is very easy decay postharvest due to disease and spoilage that are known mainly cause by microorganisms species during storage time. Therefore, fungal and bacterial species that may cause decay on postharvest guava was isolated and evaluated its the antimicrobial and antifungal ability by low-molecule-weight (LMW) chitosan in combination with nano-silicon dioxide (nano SiO_2) compound was carried out. The study successfully isolated four fungal species, namely *Chrysosporium tropicum*, *Cladosporium sphaerospermum*, *Aspergillus wentii*, *Colletotrichum acutatum* and three bacterial species, namely *Azotobacter* sp., *Escherichia coli*, *Bacillus subtilis,* which is most likely to cause decay on postharvest guava. It is found that a mixture of 0.04% nano SiO_2 and 1% LMW chitosan 44.5 kDa are capable confront microorganism tested with the highest antibacterial zone diameter and the lowest diameter of

growing fungi. This compound used in storage guava to control disease and prolong shelf-life postharvest guava.

Keywords: Chitosan, nano-silicon dioxide, antimicroorganism ability, postharvest guava

Introduction

The guava (*Psidium guajava* L.) is one of the most popular tropical climacteric fruits and commonly eaten fresh, contains extremely nutritious as well as substances with natural biological activities (Pal *et al*., 2004). In Vietnam, guava is grown popular in southern provinces, especially, Taiwan guava is most grown due to its high quality and productivity, sweet taste, crispy, and spongy. However, easy spoilage and short shelf-life are negative features of ripening guava, resulting in long-distance transportation (Hong *et al*., 2012). It was reported that attacking by microorganisms caused spoilage including *Bacillus* sp*., Listeria* sp*., Staphylococcus* sp*., Vibrio* sp*., E.coli, Pseudomonas* sp*., Aeromonas* sp*., Colletotrichum gloeosporioides*.

Chitosan is a natural edible biopolymer, biodegradable, antimicrobial and antifungal (Rabea *et al*., 2003; Kim *et al*., 2006; Aider, 2010), antitumoural (Qin *et al*., 2002), immune regulatory (Mei *et al.,* 2013), antidiabetic (Karadeniz and Kim, 2014) activities. Chitosan has been used as a coating material in many fields such as biotechnology, agricultural medicine, bio-medicine, pharmaceutical, chemical, food industries, and environmental protection (Shahidi and Synowiecki, 1991; Peniche-covas *et al*., 1992; Yamada *et al*., 1993; Felt *et al*., 1998; Ravi Kumar, 2000; Drevinskas *et al*., 2017; Wang *et al*., 2017). Moreover, chitosan is also efficiently resistive to bacteria, fungi, filamentous fungi, and yeasts which are responsible for plant diseases and spoilage in various fruits and vegetables (Franklin and Snow, 1981; Kendra and Hadwiser, 1984; Hirano and Nagao, 1989; El Ghaouth *et al*., 1992; Jeon *et al*., 2001; Bautista-

Baños *et al*., 2013; Wang *et al*., 2017), particular with *Rhizopus stolonifera* (Hernandez-Lauzardo *et al*., 2008), *Penicillium digitatum* (Chien *et al*., 2007), *Nigrospora sphaerica*, and *Fusarium culmorum* (Xing *et al*., 2016), *Colletotrichum musae* (Xiangchun *et al*., 2012). Low-molecular-weight (LMW) chitosan has been used to inhibit the growth of some positive and negative Gram bacteria (Jeon *et al*., 2001), antifungal agent (Dutta *et al*., 2012; Kulikov *et al*., 2014), reducing of postharvest damage of fruits and vegetables (Benhamou, 1996; Mlikota Gabler and Smilanick, 2001; Romanazzi *et al*., 2002; Liu *et al*., 2007), fresh food storage (Aider, 2010) as restrict lipid peroxidation on salmon (Kim and Thomas, 2007), guava storage (Hong *et al*., 2012; Krishna and Rao, 2014).

Nano-silicon dioxide (Nano SiO_2) has been widely used in water treatment, construction material, manure, and cosmetics. Recently, nano SiO_2 can be produced naturally by sol-gel methods with low cost and environment friendly (Zhang and Yang, 2008). Nano-SiO_2 can improve material properties (Lai *et al*., 2006; Yeh *et al*., 2007; Dhanasingh *et al*., 2011) and for the best properties is at 0.04% (Sun *et al*., 2016), especially, it in combination with chitosan to enhance chitosan activities applied in food storage (Yu *et al*., 2012). The combination of chitosan and nano SiO_2 is applying in extensive fields (Witoon *et al*., 2009). Thanks to the creation of hydro-associated with chitosan molecule, this hybrid compound enhanced permeability and mechanical properties (Yu *et al*., 2012; Shi *et al*., 2013), improved film formation as well as a semipermeable coating for chitosan (Silva *et al*., 2011). This combination can also increase the anti-microorganism capacity of the film (Dhanasingh *et al*., 2011), and inhibit disease on agricultural products (Silva *et al*., 2011; Yan *et al*., 2011; Sun *et al*., 2016) such as *Penicillium digitatum, Penicillium italicum, Botrydiplodia lecanidion, Botrytis cinerea*. Nearly, 1% chitosan in combination with 0.04% nano SiO_2 compounds was applied in the fruit storage (Yan *et al*., 2011; Yu *et al*., 2012) for very well efficiency.

Even though excellent reported characteristics regarding the combination of chitosan and nano-SiO2, there is not any study conducted to find out detail impact of this mixture on the

improvement of preservation for postharvest guava, especially anti-microorganisms capacity. Therefore, in this study, the impact of chitosan and nano-SiO_2 mixture to microorganisms causing spoilage postharvest guava was carried out with the purpose to find out the suitable concentration of chitosan and nano-SiO_2 for postharvest guava preservation that would broaden the fresh fruits consuming markets as well as facilitate abroad export.

Materials and methods

Isolation of bacterial and fungal causing damage postharvest guava

Taiwan guava was grown in Cai Be district, Tien Giang province, harvested after fully blossomed 75 ± 2 days, grouped as uniform size and form regardless of the sign of impact injury and disease. Then, they were transported to the laboratory, washed with distilled water. Each fruit was caused damaged by sterilized 11 blades on its skin and stored at room temperature until there was a sign of damage. These damaged positions were then removed from the fruits, left in 250 ml erlenmeyer flasks containing 40 ml sterilized distilled water. Then they are shaken in 20 mins and cell supernatant obtained after precipitaed. This solution was then diluted to concentration 10^{-1} to 10^{-3}. 0.1 ml diluted solution was spread on tryptic soy agar (TSA), potato–dextrose–agar (PDA) plates medium (Merck), and incubated in 8 days to obtain pure cultures. Then species of bacteria and fungi are sent to Bio-Techem Co., Ltd. to determine identify species.

Assay of in-vitro anti-microorganism activities of SiO_2 nano-particle and low-molecular-weights chitosan

Chitosan with LMW of 16, 44.5, 80, and 109 kDa, degree of deacetylation > 80% and nano SiO_2 with the size of 20-30 nm were purchased from the Center for Radiation Technology Research and Development Ho Chi Minh. 1% (w/v) each LMW chitosan sample was dissolved

in 1% acetic acid (v/v) and then adjusted to 5.5 pH with continuous stirring to obtain a homogeneous chitosan solution. The solution was then added with 0.04% (w/v) nano SiO_2,adjusted to 5.5 pH to obtain a uniform mixture. The mixture should be used within 1 hour to avoid SiO_2 precipitation.

The experiment was designed to randomized complete containing 10 treatments with triplicate (5 plate petries per replication) follow by chitosan LMW 16; 44.5; 80 and 109 kDa (1%); 0.04% nano SiO_2 *alone and in the* combination of each chitosan LMW 16; 44.5; 80 and 109 kDa (1%), and the control.

Antibacterial activity was assessed by disk diffusion method (Chand, 2013; Senguttuvan *et al*., 2013). Tested bacteria were prepared to spread on TSA media. Sterilized impregnated papers (6 mm in diameter) were used to spread on the plates. Then 10 µl of tested solution dispense into the middle of impregnated papers, sterilized distilled water used as the negative control. Plates were incubated at 32ºC in 24 hours. Aftermath, the diameter antibacterial zones were measured and determined by following formula: $\Delta D = D - d$ (mm). Where, D is the diameter of the antibacterial zone (mm), d is the diameter of sterile impregnated papers (mm).

Antifungal activity was assessed by by agar-well diffusion method (Chand, 2013). PDA media containing tested solution was prepared and then perforated with 6 mm diameter into in the middle of the plates containing pure growth of fungi tested, sterilized distilled water used as the negative control. Plates were incubated at 32ºC in 7 days to observe and measure the growth rate of fungi. Diameter of formulated fungi was determined by following formula: $\Delta A = A - a$ (mm). Where, A is the diameter of formulated fungi (mm); a is the diameter perforated agar (mm).

Statistical analysis

All data were analyzed by JMP 10.0 software (SAS Institute Inc., Cary, NC, USA). Significant differences between treatments were showed through Duncan test ($p < 0.05$).

Results and discussion

Isolation of bacterial and fungal causing damage postharvest guava

We are carried out isolation Taiwan guava after damage and determined four fungal species, namely *Chrysosporium tropicum* (*Ch. tropicum*), *Cladosporium sphaerospermum* (*C. sphaerospermum*), *Aspergillus wentii* (*A. wentii*), *Colletotrichum acutatum* (*C. acutatum*) and three bacterial species, namely *Azotobacter* sp., *Escherichia coli* (*E. coli*), *Bacillus subtilis* (*B. subtilis*) which causes damage postharvest guava.

Characteristic of fungal species was then described on PDA media, with regard to *Ch. tropicum* fungi has in form of fibril, flaked, slow growth, while color hyphae (Fig. 1a). *C. sphaerospermum* has in form of the fibril, grew dense, slow growth, on the top face of the fungi having deeper green color, under face having russet color, having wrinkle not smooth (Fig. 1b). *Aspergillus wentii* has in form of fibril, on the top face and bottom face of the fungi having light brown-yellow color (Fig. 1c), *C. acutatum* has in form of fibril, on the top face and bottom face of the fungi was while color (Fig. 1d) Characteristic of bacterial species was then described on TSA media: *Azotobacter* sp. colony has in form of circle, pale yellow, polished Viscid (Fig. 1e). *E. coli* colony has in form of circle, white opaque color, a polished and wetting surface on TSA media (Fig. 1f). *B. subtilis* colony has in form of circle, the edge of the crenated irregular pale yellow-brown color surface and lined on TSA media (Fig. 1g)

Testing of antibacterial activity of nano SiO_2 and low molecule weight chitosan mixture in in-vitro

The outcome of the antibacterial ability of the LMW chitosan alone or in the combination of and nano SiO_2 on *E. coli*, *Azotobacter* sp., *B. subtilis* are shown in Table 1 and Fig. 2. All the treatments applied with nano-SiO_2 and LMW chitosan mixture result in the potential antibacteria of *E. coli* and *Azotobacter* sp., *B. subtilis*, and shown a significant difference to the control

treatment ($P < 0.05$). nano SiO_2 and 44.5 kDa chitosan mixture exhibited the best effect with diameters of 12.86 ± 0.55 mm; 11.19 ± 0.56 mm; 7.86 ± 0.05 mm, respectively (Table 1, entry 6).

On the plates of *E. coli*, Entry 6 shown a significant difference with Entry 4 which treated with nano SiO_2 and 16 kDa chitosan mixture (11.61 ± 1.09 mm). On the plates of *Azotobacter* sp., Entry 4 containing nano SiO_2 and 16 kDa chitosan mixture (9.81 ± 0.24 mm) after standing Entry 6. On the plates *B. subtilis*, Entry 6 was not significant difference than Entry 5 containing 44.5 kDa chitosan mixture (7.14 ± 0.18 mm).

The results showed antibacterial ability of *B. subtilis* (the positive Gram bacteria) lower than *E. coli* and *Azotobacter* sp. (the negative Gram bacteria), and these are in agreement with the study of Jeon *et al*. (2001); No *et al.* (2002); Coma *et al*. (2003); Chung *et al.* (2004); Dutta *et al*. (2009), mentioned that antibacterial ability of negative Gram bacteria is higher than positive Gram bacteria due to its higher hydrophilicity and negative charge ability on the cell surface of Gram-negative bacteria higher, results in more chitosan adsorbed (Chung *et al*., 2004). Meanwhile, according to Zhong *et al*. (2008) reported that Gram-positive bacteria were more susceptible due to barrier ability of outer membrane of the Gram-negative bacteria.

All the treatments containing nano SiO_2 and LMW chitosan mixture showed better antibacterial activity than the one with LMW chitosan alone with identical molecular weight which can be explained by an interaction of nano-silicon dioxide and chitosan to enhance the anti-microorganism ability through improve the properties of films (Dhanasingh *et al*., 2011) and to inhibit disease on agriculture products (Yan *et al*., 2011). Due to highly porous structure of chitosan and nano silica compounds via formation of the Si–O–C bonds and hydrogen (N:H) bonds that silica was uniform dispersed and diffused in matrix of chitosan (Yeh *et al*., 2007; Dhanasingh *et al*., 2011) and lead to tightly compacted making improve the mechanical and biological properties of chitosan material (Lai *et al*., 2006).

According to Jeon *et al*. (2001), chitosan with different molecular weights expressed potential inhibition on the growth of bacteria differently and partially depend on the degree of polymerization, acetylation, bacterial species, etc. LMW chitosan can be modified permeability of bacteria film and hinder their metabolism via associated with DNA of bacteria and prevent the entry of materials or leakage of cell components to bactericidal due to its penetration into the cell bacteria (Sudarshan *et al*., 1992; Kim and Rajapakse, 2005) results in inhibition ability synthesis of mRNA and protein (Hadwiger *et al*., 1986; Sudarshan *et al*., 1992; Sebti *et al*., 2005) and excellent metal-binding capacities (Helander *et al*., 2001).

Testing of antifungal activity causing damage postharvest guava of nano SiO_2 and low molecular weight chitosan mixture in in-vitro

The results of antifungal ability of samples with only LMW chitosan or in combination with nano-SiO_2 on *Ch. tropicum*, *C. sphaerospermum*, *A. wentii, C. acutatum* are shown in Table 2 and Figures 3. All treatments which contain nano-SiO_2 and LMW chitosan mixture showed a potential antifungi of *Ch. tropicum, C. sphaerospermum, A. wentii, C. acutatum* and the results are significantly distinguished to the control treatment ($P < 0,05$). Therein, the treatment with 0.04% nano SiO_2 and 44.5 kDa chitosan mixture also showed the best antifungal ability on *Ch. tropicum, C. sphaerospermum, C. acutatum* with lowest growth fungi diameter of 6.32 ± 0.27 mm, 0.00 mm, 0.00 mm, respectively. However, with regard to *A. wentii* the plates treated with nano SiO_2 and 44.5 kDa chitosan mixture have diameter growth of fungi (9.34 ± 0.69 mm) higher negligible than that with 44.5 kDa chitosan (6.65 ± 0.35 mm). These results are corresponding to the previous studies which mentioned that LMW chitosan can be inhibited anthracnose disease caused by *Colletotrichum* sp (Xiangchun *et al*., 2012).

Most of the treatment with nano SiO_2 and LMW chitosan mixture shown better antifungal ability than those with chitosan alone at identical molecule weight due to improve properties of films that explained similar to as antibacterial activity. Chitosan has been demonstrated ability

against several fungi (Stössel and Leuba, 1984; Hirano and Nagao, 1989; Kendra *et al*., 1989). chitosan's antifungal mechanism has been explained from previous studies. On cell surfaces of fungi, polycationic polysaccharides interaction with anionic sites leads to altering membrane permeability and internal osmotic imbalance (; Leuba and stossel, 1986; Garrido Assis and de Britto, 2011), or inhibit mRNA and protein synthesis (Hadwiger and Loschke, 1981; Zhang *et al*., 2011), diffuse inside hyphae inhibit on the enzymes activity leads to inhibit the fungus growth and act more quickly than on bacteria (Cuero, 1999). Different penetration of chitosan into the fungal cell are also reported by Li *et al*. (2011), the LMW chitosan (50 kDa) penetrated very easy into cell of *Fulvia fulva*, LMW chitosan 499 kDa penetrated the inner hyphae of *Fulvia fulva* but 1320 kDa are not.

Conclusions

The study results in an obvious effect of LMW chitosan in combination with nano SiO_2 on anti-microorganisms efficiency that mainly cause decay of postharvest guava fruit. With four fungal species, namely *Chrysosporium tropicum*, *Cladosporium sphaerospermum*, *Aspergillus wentii*, *Colletotrichum acutatum* and three bacterial species, namely *Azotobacter* sp., *Escherichia coli*, *Bacillus subtilis* was isolated from damage of guava fruit. In addition, chitosan with LMW of 44.5 kDa (1%) in combination with 0.04% nano-SiO_2 shown the best capability to inhibit activity of four fungi species, i.e. *Ch. tropicum, C. sphaerospermum, A. wentii, C. acutatum*, and three bacteria species, i.e. *Azotobacter* sp., *E. coli*, *B. subtilis* on postharvest guava.

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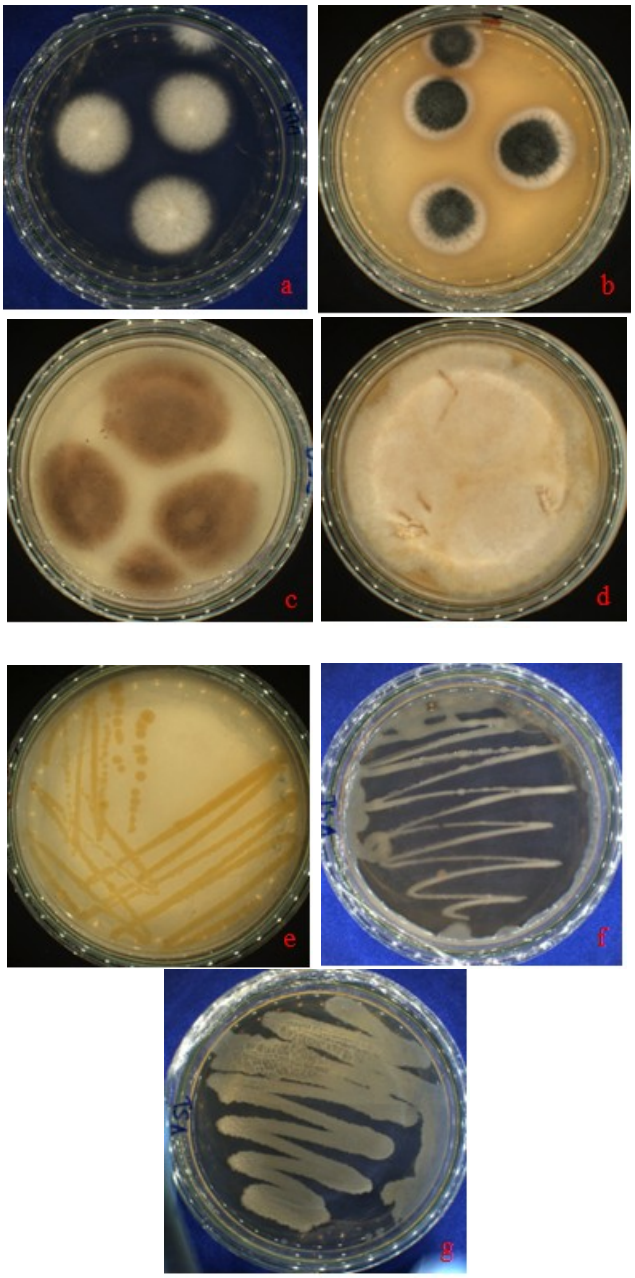
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Fig 1. Properties of fungal and bacterial species isolated on damage guava fruits: a - *Ch. tropicum; b - C. sphaerospermum; c - A. wentii; d - C. acutatum;* e - *Azotobacter* sp.*; f - E. coli; g - B. subtilis.*

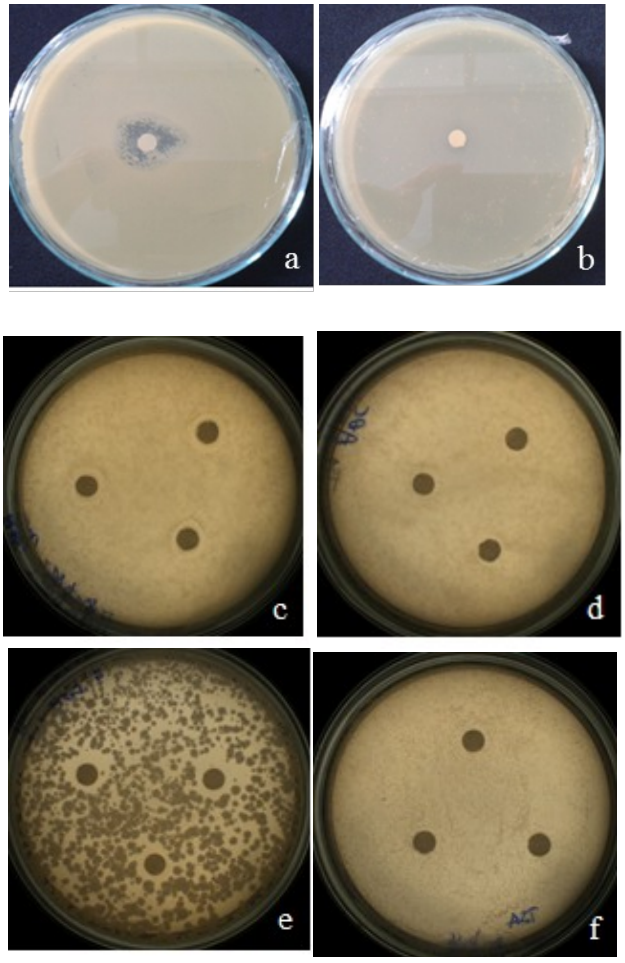

Fig 2. Inhibition ability of 44.5 kDa chitosan and 0.04% nano SiO_2 mixture against *E. coli* (a: tested sample; b: control sample), *Azotobacter* sp. (c: tested sample; d: control sample), *B. subtilis* (e: tested sample; f: control sample) in TSA medium after 24hrs at 32 ºC:

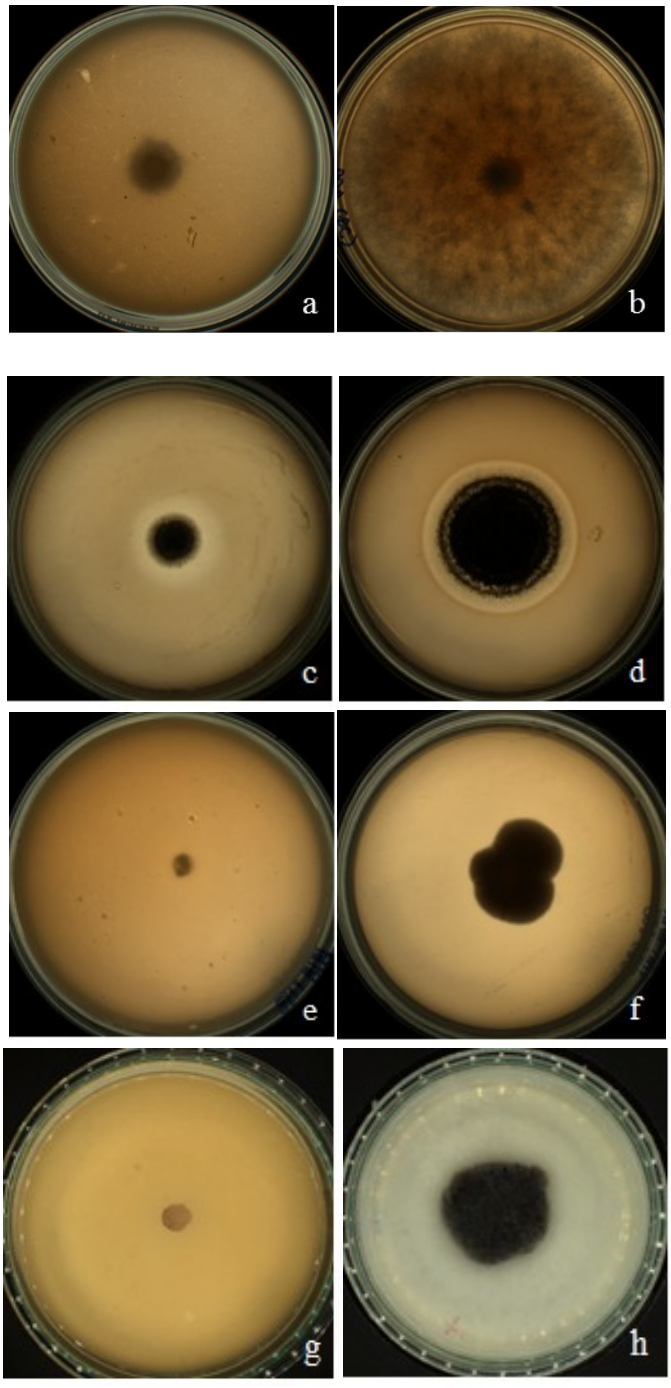

Fig 3. Inhibition ability of 44.5 kDa chitosan and 0.04% nano SiO_2 mixture in PDA medium after 5 days at 32 ºC against *Ch. tropicum* (a: tested sample; b: control sample), *A. wentii* (c: tested

441sample; d: control sample), after 7 days at 32 ºC against *C. sphaerospermum* (e: tested

442sample; f: control sample), *C. acutatum* (g: tested sample; h: control sample).

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444**Table 1.** Antibacterial activity of nano SiO_2 and LMW chitosan in *in vitro*

Entry	Compounds	Antibacterial zone diameter (mm)		
		E. coli	***B. subtilis***	***Azotobacter* sp.**
1	109 kDa LMW Chitosan	$3.45^{f} \pm 0.49$	+	$4.27^{f} \pm 0.23$
2	109 kDa LMW Chitosan and 0.04 % Nano SiO_2	$4.80^{ef} \pm 0.28$	$1.24^{e} \pm 0.12$	$5.56^{e} \pm 0.47$
3	16 kDa LMW Chitosan	$9.85^{c} \pm 0.36$	$5.07^{c} \pm 0.17$	$7.82^{c} \pm 0.36$
4	16 kDa LMW Chitosan and Nano SiO_2	$11.61^{ab} \pm 1.09$	$5.86^{b} \pm 0.16$	$9.81^{b} \pm 0.24$
5	44.5 kDa LMW Chitosan	$10.63^{bc} \pm 0.53$	$7.14^{a} \pm 0.18$	$9.39^{b} \pm 0.49$
6	44.5 kDa LMW Chitosan and Nano SiO_2	$12.86^{a} \pm 0.55$	$7.16^{a} \pm 0.23$	$11.19^{a} \pm 0.56$
7	80 kDa LMW Chitosan	$5.23^{d} \pm 0.43$	$3.65^{d} \pm 0.43$	$6.31^{de} \pm 0.17$
8	80 kDa LMW Chitosan and Nano SiO_2	$6.34^{de} \pm 0.50$	$3.97^{d} \pm 0.54$	$6.82^{cd} \pm 0.22$
9	Control	+	+	+
10	Nano SiO_2	+	+	+

445In the same columns followed by the same letter are not significantly different at confidence

446interval 95%; +: not inhibited.

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457**Table 2**. Antifungal activity of nano SiO_2 and LMW chitosan in *in-vitro*

Entry	Compounds	Diameter of growing fungi (mm)			
		Ch. tropicum	***C. Sphaerospermum***	***A. wentii***	***C. acutatum***
1	109 kDa LMW Chitosan	$16.76^{d} \pm 0.79$	$6.88^{b} \pm 0.58$	$14.64^{ef} \pm 0.39$	$14.31^{e} \pm 1.04$
2	109 kDa LMW Chitosan and 0.04% Nano SiO_2	$14.47^{c} \pm 0.87$	++	$13.57^{de} \pm 0.43$	$13.05^{de} \pm 0.27$
3	16 kDa LMW Chitosan	$12.56^{b} \pm 0.70$	++	$10.93^{bc} \pm 0.82$	$8.22^{c} \pm 0.68$
4	16 kDa LMW Chitosan and 0.04% Nano SiO_2	$7.24^{a} \pm 0.79$	++	$12.09^{cd} \pm 0.57$	$5.42^{b} \pm 1.05$
5	44.5 kDa LMW Chitosan	$8.04^{a} \pm 0.61$	++	$6.65^{a} \pm 0.35$	++
6	44.5 kDa LMW Chitosan and 0.04% Nano SiO_2	$6.32^{a} \pm 0.27$	++	$9.34^{b} \pm 0.69$	++
7	80 kDa LMW Chitosan	$16.05^{cd} \pm 0.71$	++	$16.57^{fg} \pm 0.39$	$11,76^{d} \pm 0,55$
8	80 kDa LMW Chitosan and 0.04% Nano SiO_2	$14.56^{c} \pm 0.46$	++	$18.36^{g} \pm 1.05$	$11,18^{d} \pm 0,54$
9	Control	$80.00^{e} \pm 0.00$	$29.17^{c} \pm 1.76$	$29.87^{h} \pm 1.45$	$18.44^{f} \pm 1.18$
10	0.04% Nano SiO_2	$80.00^{e} \pm 0.00$	$29.30^{c} \pm 1.27$	$30.28^{h} \pm 0.95$	$18.13^{f} \pm 0.83$

458In the same columns followed by the same letter are not significantly different at confidence

459interval 95%; ++: completely inhibited.

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