

Research Article

Synthesis of Chitosan Oligomers/Propolis/Silver Nanoparticles Composite Systems and Study of Their Activity against *Diplodia seriata*

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The synthesis and characterization of composites of oligomeric chitosan with propolis extract which allow the incorporation of a third component (silver nanoparticles) are reported, together with their application in aqueous or hydroalcoholic solutions with a view to the formation of adhesive substances or nanofilms for the protection of vineyards against harmful xylophagous fungi. The antimicrobial properties of the association of the two biological products or those resulting from the incorporation of silver nanoparticles (NPs) are studied and discussed. The efficacy of the chitosan oligomers/propolis/silver NPs ternary system is assessed *in vitro* for *Diplodia* fungi. A preliminary study on the convenience of replacing propolis with gentisic acid is also presented.

1. Introduction

With regard to hybrid materials, there is a growing realization of the importance of focusing on low-cost, environmentally friendly biopolymers. The natural, biodegradable, and biocompatible chitosan (glucosamine polymer with β -1,4 bonds), formed by the alkaline N-deacetylation of chitin, is one of the most promising candidates, due to its ability to form films, transparency, nontoxicity, excellent adsorption features, and so forth [1].

In addition, chitosan has been found to have antimicrobial properties [2, 3], in which the size of its oligomers plays a key role [4]. For example, heptamers and higher oligomers induce an increase of pisatin, an antifungal substance from *Pisum sativum* L. [5]. It has also been demonstrated that

polymeric chitosan and chitosan oligomers induce phytoalexins or antimicrobial compounds that help limit the spread of pathogens [6]. Chemical synthesis of different sizes of chitosan oligomers with specific biological activity has been described by Kuyama et al. [7].

Several possible mechanisms have been proposed to explain the antibacterial properties of chitosan: it is known that positively charged amine groups are capable of interacting with the negatively charged bacterial cell membrane and, in addition, chitosan may also bind to DNA, leading to inhibition of mRNA and proteins synthesis [8].

On the other hand, other substances—such as propolis—are also known to have antimicrobial properties and are extensively used in traditional medicine [9, 10]. Propolis is a natural resinous hive product collected by honeybees from

various plant sources [11] which contains over 150 chemical species (such as coumarins, flavonoids, polyphenols, phenolic aldehydes, sesquiterpene quinines, amino acids and steroids) [12]. Its strong antimicrobial activity may be due to the high content of total phenols and flavonoids [9]. Propolis has also been found to have applications as an antioxidant and in food preservation [13].

Silver nanoparticles have been deemed as one of the most promising antimicrobial species from a nanotechnology-based approach, since their activity is very broad and is well above that of raw silver. For example, silver ions can bind to negatively charged bacterial peptidoglycan walls and can diffuse into bacterial cells and bind to DNA bases, leading to bacterial death and/or inhibiting the replication and transcription processes and preventing further bacterial production [14]. Moreover, the generation of reactive oxygen species, which leads to nanotoxicity processes, is also a well-established antimicrobial mechanism. The main disadvantages that limit the use of nanosilver are its ease of aggregation and the uncontrolled release of silver ions and their cytotoxicity potential [15].

The combination of polymers and nanosilver may synergistically improve their antimicrobial effects, and the use of *in situ* synthesis methods allows its incorporation into the polymer matrix attaining uniform distributions and avoiding aggregation. We herein report a facile synthesis procedure based on a sonochemistry approach for the preparation of a new ternary composite material—which consists of chitosan oligomers, propolis, and silver NPs—and study its antimicrobial properties against *Diplodia seriata* fungus.

It is known that the xylophagous fungi that are present in greater proportion and that irremediably cause plant death of the vine are, firstly, *Diplodia seriata*, followed by *Phaeoacremonium aleophilum*, *Cylindrocarpon* spp., *Fomitiporia punctata* Murrill, *Fomitiporia mediterranea*, *Phaeoconiella chlamydospora*, *Botryosphaeria dothidea*, *Stereum hirsutum* and *Eutypa lata* [16]. This disease is considered highly virulent because the infection is caused by fungi entry through wounds caused by vine pruning [17–19].

In the literature, Prakongkha et al. [20] proposed the use of bare chitosan in vineyards; Matei et al. reported that chitosan can inhibit *esca* (a vine disease that ravaged vineyards from Cognac to Bordeaux, France) mycelia growth by 90% [21]; and Aziz et al. [22] successfully demonstrated that octameric chitosan oligomers doped with copper sulfate can induce defense reactions of the vine and resistance to gray mold and mildew. Other authors have assessed the antimicrobial properties for chitosan/nanosilver [23–26]. Nonetheless, to the best of the authors' knowledge, no studies have been conducted on ternary composites. In this paper, we assess how the presence of chitosan oligomers—to which phenolic compounds such as phenolic acids and/or flavonoids have been incorporated—affords the composite with antioxidant and soluble compounds that can interact to form chelates or bridges with inorganic species in aqueous solution, such as silver NPs, leading to novel antimicrobial agents, without posing any danger to the plant or to the substrate wherein the composite material is applied. The association of the three components attempts to further improve the antimicrobial

performance of their active principles in comparison with their separate application.

2. Materials and Methods

2.1. Reagents and Characterization Equipment. Medium molar mass chitosan (CAS number 9012-76-4) was purchased from Sigma Aldrich Química SL (Madrid, Spain) and from Hangzhou Simit Chemical Technology Co., Ltd. (Hangzhou, China). Propolis came from Burgos region (Spain), in the Duero river basin, and has a polyphenols and flavonoids content of ca. 10% wt/v. Silver nitrate (CAS number 7761-88-8) and malt extract agar (Reference 105398) were supplied by Merck Millipore (Darmstadt, Germany). Potassium methoxide solution (25 wt.% in methanol, CAS number 865-33-8) and ethanol (puriss. p.a., ACS reagent, CAS number 64-17-5) were also purchased from Sigma Aldrich Química SL. Genticic acid (CAS number 490-79-9) was supplied by Shanghai Vincor BioEngineering Co., Ltd. (Shanghai, China). The isolated *Diplodia seriata* mycelium (Y207 1-1c) was supplied by ITACYL (Castilla y León, Spain).

An ultrasonic machine, model CSA 20-S500, 20 KHz has been used for solutions sonication.

X-ray powder diffractograms of the samples were obtained using a Bruker D8 Advance Bragg-Brentano diffractometer, in reflection geometry.

Infrared spectra were recorded with a Thermo Nicolet 380 FT-IR apparatus equipped with a Smart Orbit Diamond ATR system, in order to identify the chemical functional groups.

Optical absorption spectra of the silver NPs in the UV-Vis region were recorded with a Shimadzu UV-2450 UV-Vis spectrophotometer.

Scanning electron microscopy (SEM) images were collected with a FEI-Quanta 200FEG equipped with a Genesis energy-dispersive X-ray (EDS) spectrometer system. Transmission electron microscope (TEM) micrographs were collected with a JEOL JEM-FS2200 HRP equipped with an Oxford Instruments INCA Energy TEM 250 EDS probe.

2.2. Culture Media and Activity Assays. The fungicidal action of the products under study was tested *in vitro* using malt extract agar (MEA) as a culture medium. In the study on the fungus, previously isolated *Diplodia seriata* mycelia (Y207 1-1c) were used, which had been replicated in Petri dishes two weeks in advance at 25°C. From the periphery of the pure culture, 7 mm diameter disks were cut and then transplanted into Petri dishes prepared with MEA in combination with one of the products under study (namely, bare chitosan oligomers, propolis, chitosan oligomers/propolis, chitosan oligomers/silver NPs, and chitosan oligomers/propolis/silver NPs). For the preparation of the base medium, 35 g of MEA was dissolved in 750 mL of distilled water, to which 2 mL of antibiotic (chloramphenicol) was added to prevent bacterial contamination, and the flasks with the mixture were then sterilized in an autoclave at 100°C for 45 min. For the control, the culture medium “as-is” was placed in sterile Petri dishes (20 mL/dish) and allowed to cool before replication of the *Di. seriata* fungal mycelia. To prepare

TABLE 1: Composition of each of the solutions mixed with MEA for the antifungal activity tests.

Solution	Composition
(1) Chitosan	20 mg of chitosan/1 mL of H ₂ O
(2) Propolis	50 mg of propolis/1 mL of H ₂ O : ethanol (7 : 3)
(3) Silver NPs	170 µg/1 mL of H ₂ O
(4) Chitosan oligomers/propolis	1 : 1 ratio of solution (1) and solution (2)
(5) Chitosan oligomers/silver NPs	8 : 1 ratio of solution (1) and solution (3)
(6) Chitosan oligomers/propolis/silver NPs	8 : 8 : 1 ratio of solutions (1), (2), and (3), respectively

the Petri dishes for the different mixtures or combinations of chitosan oligomers/propolis/silver NPs, the culture medium was cooled to 50°C and, at this temperature, 1.5 mL of the different solutions (see Table 1) was mixed with 18.5 mL of MEA and poured into each Petri dish. The solutions were then allowed to cool down to room temperature (RT) prior to replication of the fungus mycelia. Growth measurements were performed in triplicate. The diameter of fungal growth was measured on a daily basis for 20 days, and the inhibition percentage (IP) was calculated taking the pure MEA culture (control) as a reference according to the following equation [27]:

$$\text{Inhibition percentage (\%)} = \frac{D_{mc} - D_{mp}}{D_{mc}} \times 100, \quad (1)$$

where D_{mc} is the diameter of the mycelium in the control (pure MEA) and D_{mp} is the diameter of the mycelium of the sample mixed with one of the antimicrobial composites.

2.3. Synthesis of Solutions and Films of Chitosan Oligomers, Chitosan Oligomers/Propolis, and Chitosan Oligomers/ Propolis/Ag NPs

2.3.1. Chitosan Oligomers Preparation. Chitosan oligomers aqueous solutions were prepared from a solution of commercial medium molar mass chitosan (with molar masses in the 190000–310000 g/mol range for the Sigma Aldrich product and with molar masses in the 140000–300000 g/mol range for the Hangzhou Simit Chemical Technology Co. product) in AcOH 2% at pHs 4–6. The hydrolysis was performed by stirring for 12 hours followed by 3–6 sonication periods (5 minutes each), at temperatures in the 30 to 60°C range and with H₂O₂ concentrations ranging from 0.3 to 0.6 M, obtaining oligomers with molar masses in the 6000 to 2000 g/mol range, respectively, in agreement with the analogous microwave-based procedure reported by Sun et al. [28]. The molar mass of the chitosan samples was determined by measuring the viscosity, in agreement with Yang et al. [29], in a solvent of 0.20 mol/L NaCl + 0.1 mol/L CH₃COOH at 25°C using an Ubbelohde capillary viscometer. Molar masses were determined using the Mark-Houwink equation $[\eta] = 1.81 \times 10^{-3} M^{0.93}$ [30].

The solutions were then decanted to remove any water insoluble material, were allowed to rest till cloudiness was observed, and were centrifuged to isolate the chitosan oligomers. These were redissolved again in AcOH 0.5%, obtaining the solutions for the assays.

2.3.2. Propolis Extraction. The propolis solution was prepared by grinding raw propolis to fine powder and subsequent extraction of the active ingredients by maceration in a hydroalcoholic solution 7:3 (v/v) for one week at room temperature. A hydroalcoholic medium was chosen over absolute ethanol because it results in wax-free tinctures containing higher amounts of polyphenolic substances [31]. The resulting solution was then percolated (1 L/min) and filtrated with a stainless steel 220 mesh to remove any residue, followed by concentration at a temperature below 60°C with ultrasound equipment to finally obtain a clarified propolis extract.

2.3.3. Silver NPs Preparation. Silver nanoparticles were prepared by a sonication method, without resorting to UV stabilization (used, e.g., in [32]), as follows: an aqueous solution of AgNO₃ (50 mM) was treated with sodium citrate (30 mM) and the resulting solution was cooled and stirred at a temperature between 5 and 10°C. Subsequently, it was deoxygenated with an inert gas (N₂) for over 30 minutes and the pH was adjusted between 7 and 8. Polyvinylpyrrolidone was added to prevent the silver nanoparticles aggregation. A 10 mM solution of NaBH₄ (reducing agent) was then added dropwise; the first droplet made the solution turn from colorless to yellowish and successive droplets led to an intensification of the yellow color (care had to be taken so as to avoid an excess of reducing agent, which would lead to a brownish color). After vigorous stirring for one hour, the yellowish solution was sonicated for 3–5 minutes and then allowed to rest and stabilize for at least 24 hours in a refrigerator at 5°C.

2.3.4. Binary and Ternary Solutions Preparation. Chitosan oligomers/propolis solution was prepared by mixing the two components in a 1:1 w/v ratio (Table 1), followed by sonication, obtaining a caramel colored gel or precipitate, similar to that obtained by Mascheroni et al. [33] using an alternative membrane-based procedure.

Ternary solutions were prepared by adding a mixture of propolis extract and silver NPs solutions to the solution containing chitosan oligomers (Table 1). A few droplets of a solution of potassium methoxide in methanol 25% were added to adjust the pH to 4–6 (the methanol which results by hydrolysis is below 10 ppm, and at this concentration it is innocuous to the fungi). The resulting solutions were stirred for 1 hour and sonicated for 2–3 minutes and remained clear. These solutions were mixed with MEA for the activity assays.

When a film was required (e.g., for SEM/TEM characterization), glyoxal 0.25% v/v was added to facilitate the interaction and the pH was adjusted to 8–9. Upon stirring and sonication, the solutions were allowed to rest for approximately 1 hour and gels appeared at the bottom of

TABLE 2: Assignment of FTIR spectra bands.

Chitosan	Chitosan oligomers	Propolis	Chitosan/propolis	Chitosan/propolis/silver NPs	Assignment
3290	3285		3285	3300	$\nu(\text{OH})$ overlapped to $\nu_s(\text{N-H})$ [35, 36]
		2970		2972	C-H bands of aromatic compounds
		2930		2929	$\nu_{\text{as}}(\text{C-H})$ in $-\text{CH}_2$ [35]
2867	—	2873		2873	$\nu_s(\text{C-H})$ in $-\text{CH}_3$ [35]
1646	1649	1640 1598		1707	$\nu(-\text{C=O})$ of the amide group CONHR in chitosan [36, 38] and $\nu(-\text{C=O})$ of flavonoids and lipids, found in propolis [39]/aromatic ring deformations [39]
1568	—		1554	1560	$\nu(-\text{C=O})$ protonated amide group [35] $\delta(\text{NH}_3)$ protonated amine group
1417	1419		1404	1409	$\delta(\text{OH})$ [40]
1372	1375	1377		1373	$\delta(-\text{CH}_3)$ [41]
		1334	1341	1333	Typical propolis C-O and C-OH vibrations [39]
1318	1317				$\nu_s(-\text{CH}_3)$ tertiary amide [35] $\omega(-\text{CH}_2) + \text{OH}$ in-plane deformation
		1284 1228		1260	$\nu(\text{C-O-H})$ [35]
1152	1148		1150	1160	$\nu_{\text{as}}(\text{CO})$ in oxygen bridge resulting from deacetylation of chitosan
		1136		1133	Alkenes bands from propolis (coumaroyl glycerol) [42]
1060	1081	1078	1066	1084	$\nu(\text{CO})$ of the ring C-O-H, C-O-C and CH_2CO [35, 39]
		1038 989		1042 986	Bands from propolis
		920	923	922	Bands from propolis
892	892			872	$\omega(\text{C-H})$ of the saccharide structure [35]
		836		835	Band from propolis
			646	656	Typical bands from binary and ternary formulations
			617	615	

the flasks. These materials were isolated from the solution, were poured in polypropylene substrates [34], and were dried under vacuum at 20°C in dry atmosphere, yielding films with thicknesses in the 0.3 to 0.6 mm range. Details on these procedures have recently been the subject of a patent (Application number ES P201431591, filing date: 30/10/2014).

3. Results and Discussion

3.1. Vibrational Characterization. The ATR-FTIR spectra of commercial chitosan, chitosan oligomers, propolis, chitosan oligomers/propolis binary composite, and chitosan oligomers/propolis/silver NPs ternary composite are depicted in Figure 1. The assigned characteristic FTIR absorption bands derived from Figure 1 are summarized in Table 2.

Analyses of both the binary chitosan-propolis formulation and the ternary composite showed that the first band in the ATR-FTIR spectra was shifted to higher wavelengths in comparison with the bare chitosan spectrum (from 3285–3290 to 3300 cm^{-1}) [35, 36], suggesting that effective hydrogen bonding occurred between chitosan and propolis. This

interaction is an indicator of a synergy between the two products.

Another significant feature for the ternary composite spectrum was an obvious shift to 1700 cm^{-1} of the band located at around 1650 cm^{-1} , accompanied by an increase in the intensity, proving the responsibility of chitosan amino group for silver envelopment [36–39]. In the same way, the change in the OH band intensity in the ternary composite spectra at 1409 cm^{-1} (assigned to the OH deformation vibrations of the secondary alcohols in the pyranose monomers) revealed that the hydroxyl groups may have also participated in the stabilization of the silver nanoparticles via interaction of Ag^+ with the electron abundant oxygen atoms of the hydroxyl groups of the chitosan. These results are in agreement with those reported by Boanić et al. [40]. Moreover, the increase in intensity of the band at 1380 cm^{-1} (attributed to CH_3 bending in NHCOCH_3 group [41]) can also be attributed to interactions with silver NPs.

In the particular case of the band at 1133 cm^{-1} , characteristic of ester groups ($-\text{C-O}$ stretching) from propolis

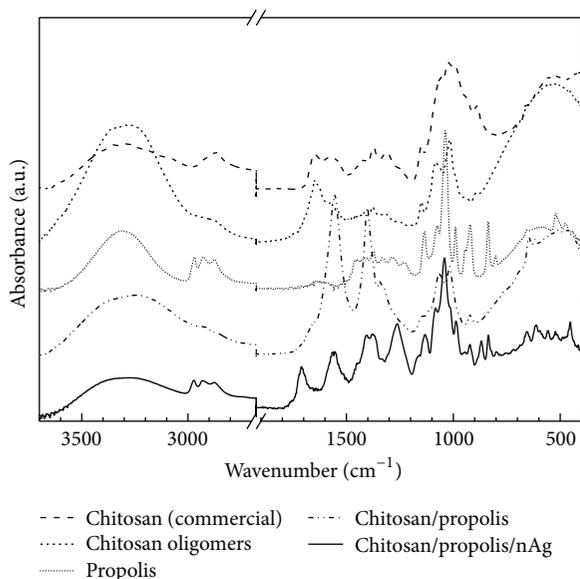


FIGURE 1: ATR-FTIR spectra of the chitosan- and propolis-based materials under study.

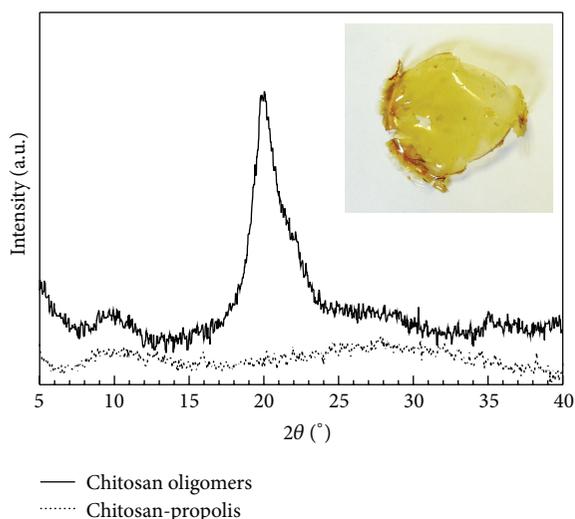


FIGURE 2: X-ray powder diffraction pattern for chitosan oligomers (solid line) and chitosan oligomers/propolis composite (dotted line). The inset shows a photograph of the chitosan oligomers/propolis film.

components [42], a change in the intensity of the band was also observed, attributable to interactions with silver NPs.

Unassigned typical bands from binary and ternary formulations have been found at around 650 and 616 cm^{-1} .

3.2. X-Ray Characterization. The X-ray diffraction study of the chitosan oligomers exhibits the expected broad peaks at $2\theta = 10^\circ$ and $2\theta = 20^\circ$ (Figure 2), in good agreement with Kumar et al. [43]. However, the peak observed for chitosan at $2\theta = 20^\circ$ disappeared and the very broad peak at $2\theta = 10^\circ$ became weak in the chitosan-propolis sample. These results suggest that chitosan has good compatibility, which leads

to the formation of a composite with an amorphous form, suitable for bioapplications [44].

3.3. Silver NPs Characterization. Silver nanoparticles were characterized by UV-Vis absorption, XRD, and TEM analysis, revealing the formation of highly pure, crystalline silver nanoparticles of ca. 30 nm (see Section 3.4). The UV-Vis spectrum (Figure 3(a)) showed the expected intense surface plasmon resonance (SPR) band at around 420 nm [45]. The X-ray powder diffraction pattern (Figure 3(b)) matched well with the standard patterns of silver (JCPDS number 04-0783). All the peaks of the pattern can be readily indexed to face-centered-cubic silver, where the diffraction peaks at 38.2, 44.5, 64.5, and 77.5° can be ascribed to the reflection of (1, 1, 1), (2, 0, 0), (2, 2, 0), and (3, 1, 1) planes, respectively.

3.4. Textural Properties. The texture of the ternary composite films has been studied by SEM and TEM. The SEM micrographs (Figure 4(a))—similar to those reported by Mohararam et al. [26]—show the good homogeneity of the materials under study, while the TEM micrograph (Figure 4(b)) allows estimating the size of the silver NPs, in the 24 to 35 nm range.

3.5. Antifungal Activity of Chitosan. As noted above, the size of the chitosan chains has a significant impact on its antimicrobial activity [4]. Consequently, prior to the evaluation of the different composites, a first study was conducted only for chitosan. Two commercial medium molar mass chitosan solutions were compared with the chitosan oligomers prepared according to the procedure described in Section 2.3.1. Whereas the chitosan oligomers led to mycelia death, the fungus was able to grow in the culture media with medium molar mass chitosan (see Figure 5). Consequently, only chitosan oligomers have been used in the studies described below.

3.6. Antifungal Activity of the Propolis, Chitosan Oligomers/Propolis, and Chitosan Oligomers/Propolis/Silver NPs Colloidal Solutions against *Diplodia seriata* Fungus. The antifungal activity has been studied for the aqueous and hydroalcoholic mixtures of bare propolis, chitosan oligomers/propolis, and chitosan oligomers/propolis/silver NPs, assessing the influence of low alcohol concentrations (ca. 5%) on the mixtures and analyzing the growth diameter of the *Diplodia seriata* mycelia. As indicated above, the assays have been conducted in triplicate biological repetitions, and all the results are in average.

For bare propolis (see Figure 6(a)), in presence of alcohol, the mycelium diameter increased exponentially in an initial stage, with a slope change on the 7th day and reaching its maximum diameter (77–78 mm) on the 13th/14th day. When alcohol was removed from the propolis extract by sonication, the activity of the fungus decreased, showing a slower and progressive growth and reaching a maximum diameter of 22 mm.

When the chitosan oligomers/propolis composite (see Figure 6(b)) was tested in the presence of alcohol, exponential

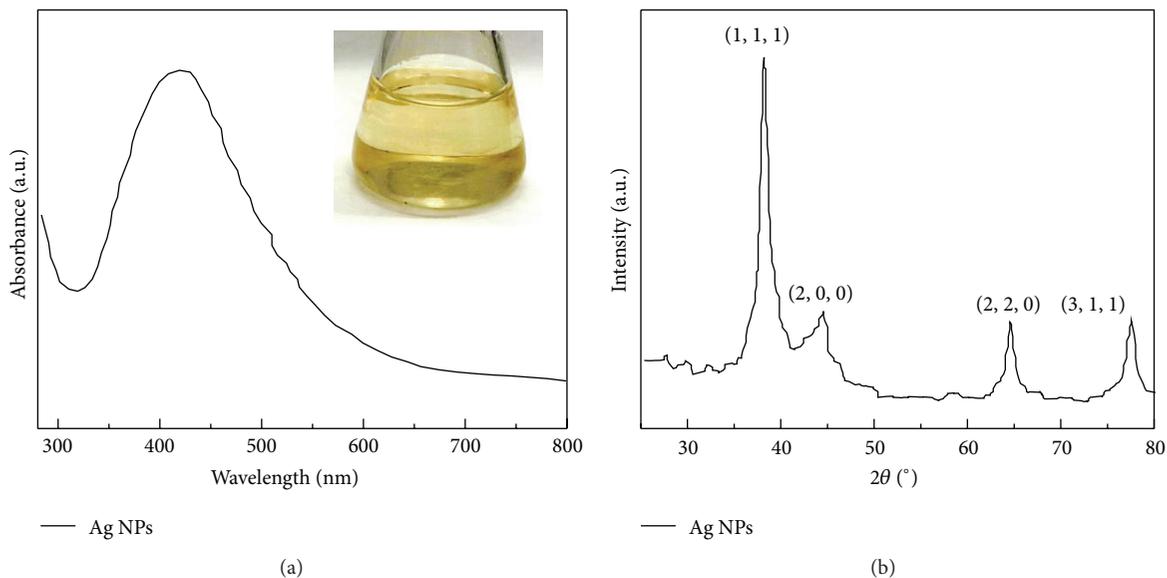


FIGURE 3: UV-Vis spectrum (a) and X-ray powder diffraction pattern, smoothed with a Savitzky-Golay 10 pt. window filter (b).

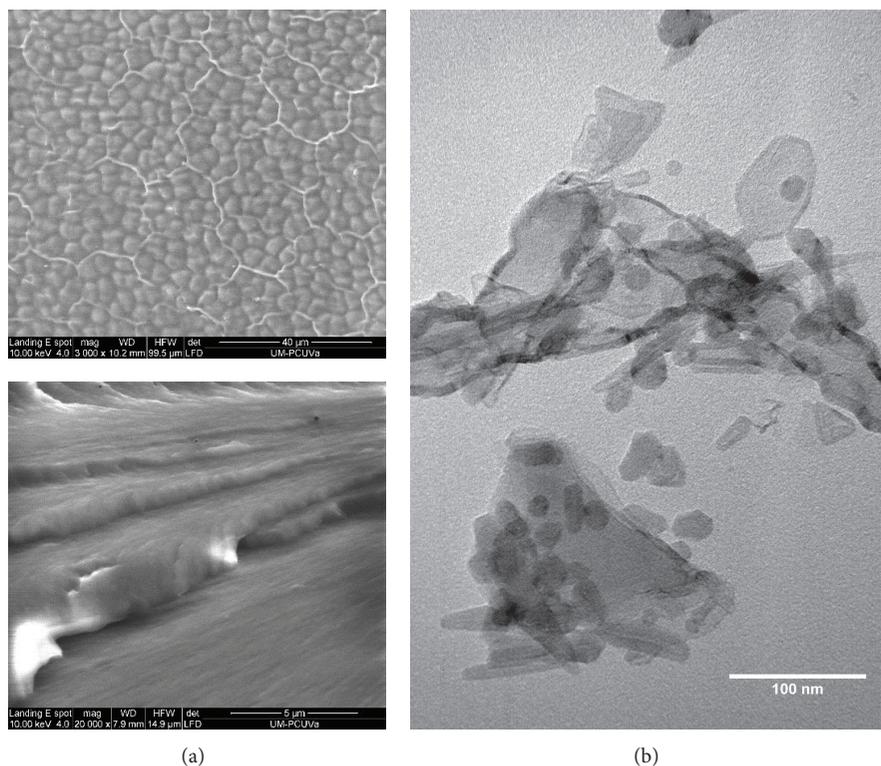


FIGURE 4: SEM micrographs (a) and TEM micrograph (b) of the ternary chitosan oligomers/propolis/silver NPs composite.

growth took place up to the 7th day, in which there was also a slope change and the growth started to be less pronounced. In comparison with bare propolis, the antifungal activity was significantly enhanced, since the maximum mycelium diameter in this case was 27 mm. Further, when alcohol was completely removed, no fungal growth took place (and the diameter remained constant at 7 mm).

A similar behavior was observed for the chitosan oligomers/propolis/silver NPs composites (Figure 6(c)); in the presence of alcohol, there was an almost linear increase in the mycelia diameter, reaching 41 mm, whereas in the absence of alcohol no fungal growth occurred. It can thus be inferred that the antifungal activity of the material with silver NPs was better than that of bare propolis but worse than that of

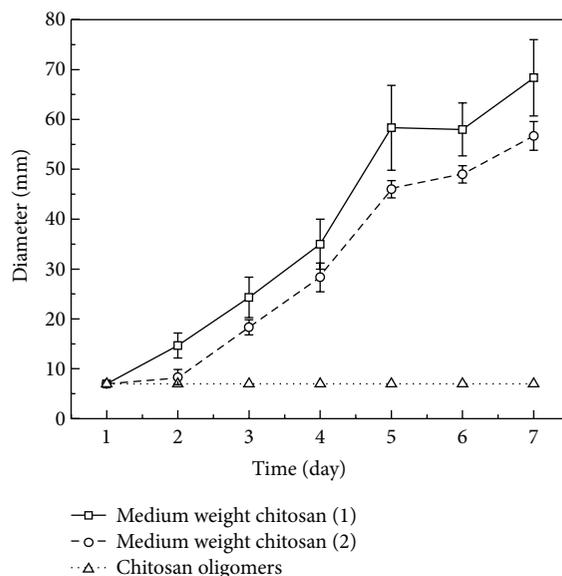


FIGURE 5: Antifungal action of medium molar mass chitosan (from Sigma Aldrich (1) and from Hangzhou Simit Chemical Technology Co., Ltd. (2)) compared to that of chitosan oligomers.

the binary composite. Nevertheless, it is worth noting that the presence of silver NPs reduced the standard deviation values and made the growth more linear (i.e., less logarithmic) in comparison with the assays conducted for bare propolis or chitosan oligomers/propolis composite in hydroalcoholic medium.

A wider comparison (summarized in Figure 7), which also includes bare chitosan (A) and chitosan oligomers/silver NPs mixture (D), further confirms previous results: the presence of alcohol significantly favored the growth of the fungus. On the contrary, when the alcohol was removed by sonication at a temperature lower than 60°C, the fungus hardly grew for bare propolis (P)—which showed an inhibition percentage of 75%—or did not grow when chitosan oligomers/propolis (B) or chitosan oligomers/propolis/silver NPs (C) composites were used. Consequently, alcohol removal from the binary or ternary mixtures is essential so as to improve the antifungal activity of these materials. This is in agreement with the findings of Zhao et al. [46], who reported that a concentration of 0.5–2% ethanol stimulated the growth of fungi, since it was partially used as a carbon source during fermentation.

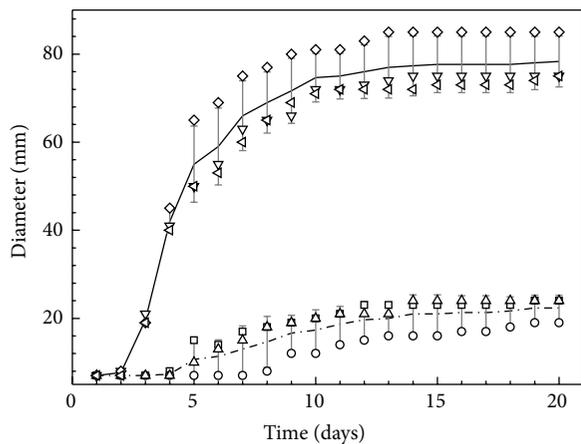
3.7. Future Lines of Research: Replacement of Propolis with Gentisic Acid. Propolis polyphenols, such as gentisic acid (2,5-dihydroxybenzoic acid) or homogentisic acid (2,5-dihydroxyphenylacetic acid), are known to have antifungal activity [47] and have been shown to increase the fungicidal activity of other chemicals (e.g., fludioxonil, a phenylpyrrole fungicide) [48]. Further, they have a widespread occurrence, being found in citrus fruits (*Citrus* spp.), grapes (*Vitis vinifera*), sesame (*Sesamum indicum*), gentians (*Gentiana* spp.), and so forth, which are amongst the probable floral origins of the Mediterranean propolis used in our study,

in agreement with Gülçin et al. [49]. Consequently, a preliminary assessment of the suitability of the former as a replacement of propolis extract has been conducted.

As it is shown in Figure 8, pure propolis, pure gentisic acid, and propolis:gentisic acid (1:1) solutions (with concentrations of 30 mg/mL for the propolis solution, 30 mg/mL for the gentisic acid solution, 1:1 ratio of the two solutions for the propolis:gentisic acid mixture, resp.) have been evaluated, and an improved performance of gentisic acid over propolis has been evidenced. Thus, an identification of the propolis components and their separate antifungal activity study needs to be conducted in future research.

4. Conclusions

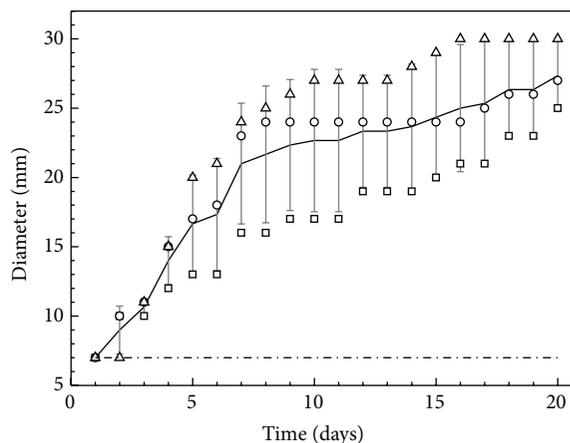
The present work reports the synthesis by a facile chemical method of a composite system consisting of a colloidal suspension of silver nanoparticles in a chitosan-propolis biopolymer matrix. The materials have been characterized by X-ray powder diffraction studies, FTIR vibrational spectroscopy, UV-Vis absorption spectroscopy, and SEM and TEM microscopies. With a view to the application of these solutions to the formation of adhesive substances or nanofilms for the protection of vineyards against harmful xylophagous fungi, the influence of low alcohol concentrations (ca. 5%) on the growth diameter of the *Diplodia seriata* mycelia has been determined. The results are conclusive on the need of alcohol removal to improve the antifungal activity of these materials. Moreover, it is essential to use low molar mass chitosan oligomers, given that their inhibitory activity is significantly higher than that of medium molar mass chitosan. Finally, it is worth noting that further research is still required so as to evaluate the separate activity of



Propolis

— Average with EtOH - - - Average without EtOH
 ▽ 1st repetition with EtOH □ 1st repetition without EtOH
 ◇ 2nd repetition with EtOH ○ 2nd repetition without EtOH
 ◁ 3rd repetition with EtOH △ 3rd repetition without EtOH

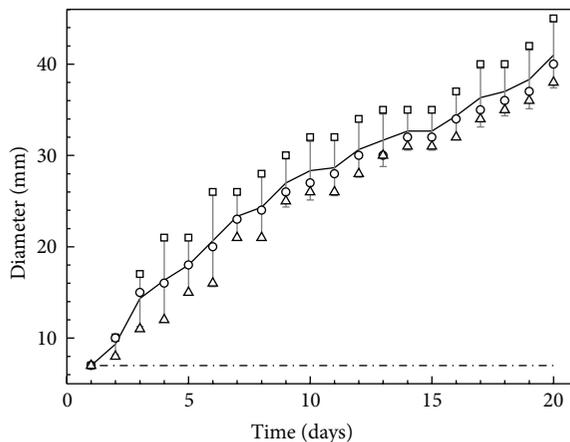
(a)



Chitosan/propolis

— Average with EtOH △ 3rd repetition with EtOH
 □ 1st repetition with EtOH - - - Average without EtOH
 ○ 2nd repetition with EtOH

(b)



Chitosan/propolis/silver NPs

— Average with EtOH △ 3rd repetition with EtOH
 □ 1st repetition with EtOH - - - Average without EtOH
 ○ 2nd repetition with EtOH

(c)

FIGURE 6: Antifungal action of the aqueous and hydroalcoholic media for bare propolis (a), chitosan oligomers/propolis (b), and chitosan oligomers/propolis/silver NPs (c) composites.

the different components of propolis, as evinced by the enhanced antifungal behavior of gentisic acid in comparison with propolis, and on the synergistic effect of the three components of the composite.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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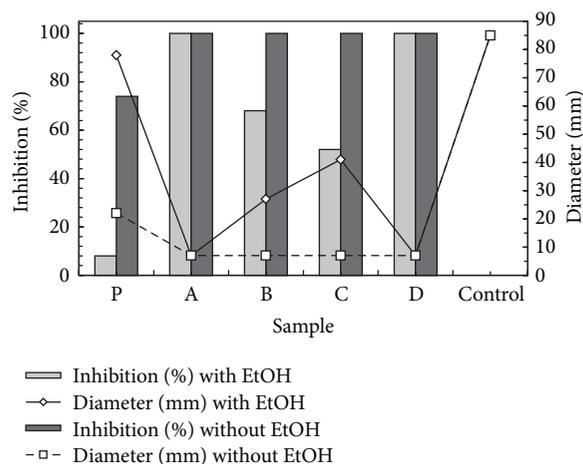


FIGURE 7: Inhibition percentage and growth diameter of the *Diplodia seriata* fungus mycelia for propolis (P), chitosan oligomers (A), chitosan oligomers/propolis (B), chitosan oligomers/propolis/silver NPs (C), chitosan oligomers/silver NPs (D), and control, in aqueous solutions (without EtOH) and in hydroalcoholic solutions (with EtOH).

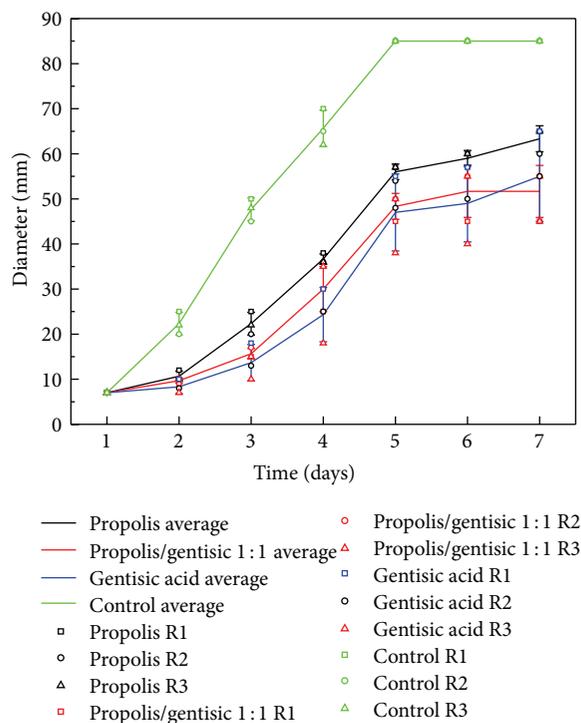


FIGURE 8: Comparison of the antifungal activity of pure propolis, a propolis : gentisic acid mixture (1:1), and pure gentisic acid. R1, R2, and R3 stand for 1st, 2nd, and 3rd repetition.

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