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Research Article

Characterization of Intact Chitosan Obtained from Bradyporus sp. (Orthoptera, Insecta) and Its Cytotoxicity on Human Cell Line (HEK293)

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Abstract
Intact chitosan film was produced from Bradyporus sp. (Orthoptera, Insecta) and characterized by Fourier Transform Infrared Spectroscopy (FTIR), Thermogravimetric Analysis (TGA), X-ray diffractometer (XRD), Scanning Electron Microscopy (SEM) and elemental analysis. MTT test was performed to determine the cytotoxic effects of chitosan. Chitosan administered at optimal and overdose intervals was applied to HEK293 cells for 24, 48 and 72 hours. In the MTT cytotoxicity test, the IC50 dose of chitosan was 1427 µM at 24 hours, 808.9 µM at 48 hours, and 611.6 µM at 72 hours. Considering all the conducted analyses and cytotoxic experiments, the obtained intact novel chitosan film from the insects could find applications for biotechnological applications.

1. Introduction

Chitin, a polysaccharide composed of N-acetyl-D-glucosamine components (poly (β-(1->4)-N-acetyl-D-glucoseamine)), is the second most abundant biopolymer on the earth. It is reported that this important biopolymer is found in nature mainly in invertebrates, arthropods, insects, algae, fungi and eukaryotic yeasts [1]. Although the most common sources of chitin in nature are indicated as shellfish and aquatic invertebrates, these are limited sources. Therefore, insects are a good source of chitin due to their ease of transportation (abundance in nature) and safe applications [2,3].

In particular, some insects with hard and robust exoskeletons, such as chewing insects (Coleoptera), may be good alternatives to shellfish, thanks to their high chitin content [4]. Therefore, chitin isolation and characterization studies have been carried out on many insect species such as Lepidoptera ((Bombyx mori [5,6], Clanis bilineata [7,8], Coleoptera (Tenebrio molitor [9], Omophlus sp. [10], Melolontha sp. [11], Hydrophilus piceus [12], Orthoptera (Dociostaurus maroccanus [13], Brachytrupes portentosus [14], Oedaleus decorus [15], Ailopus simulatrix [16], Hymenoptera (Apsis mellifera [17], Vespa crabro [17,18], Diptera (Musca domestica [19,20], Hermetia illucens [21,22], Hemiptera (Ranatra linearis [12], Cryptotympana atrata [7]), Dictyoptera (Periplaneta americana [15], Blaberus giganteus [23], Blattella germanica [24]), Odonata (Sympetrum fonscolombi [11], Anax imperator [12].

Chitin and chitin derivatives are used in food, medicine, dentistry, cosmetics and agriculture sectors due to their antioxidant activity [25,12], anti-inflammatory effect [26], anti-microbial effect [27,28] and anti-cancer effect [29,30]. In recent years, chitosan, a chitin derivative, has become one of the most popular biopolymers. Chitosan and chitooligosaccharides have been included in many biological industries (biomedical applications, drug delivery systems, food/chemistry studies), especially medicine/health, due to their non-toxicity [31].

In this study, intact chitin and chitosan films were produced from Bradyporus sp and analyzed by FTIR, TGA, XRD, elemental analysis and SEM and its cytotoxic effects on HEK293 cells were investigated for the first time.

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2. Materials and Methods

2.1. Collection of Materials

Bradyporus sp. (Orthoptera, Insecta) (Figure 1a) were collected from Aksaray, Türkiye. After collection, the samples were illustrated using a digital camera. The collected specimens were fixed in 96% alcohol against possible contamination and stored for this study. All adhering contaminant particles were separated from the body and rinsed with alcohol from the outer surface.

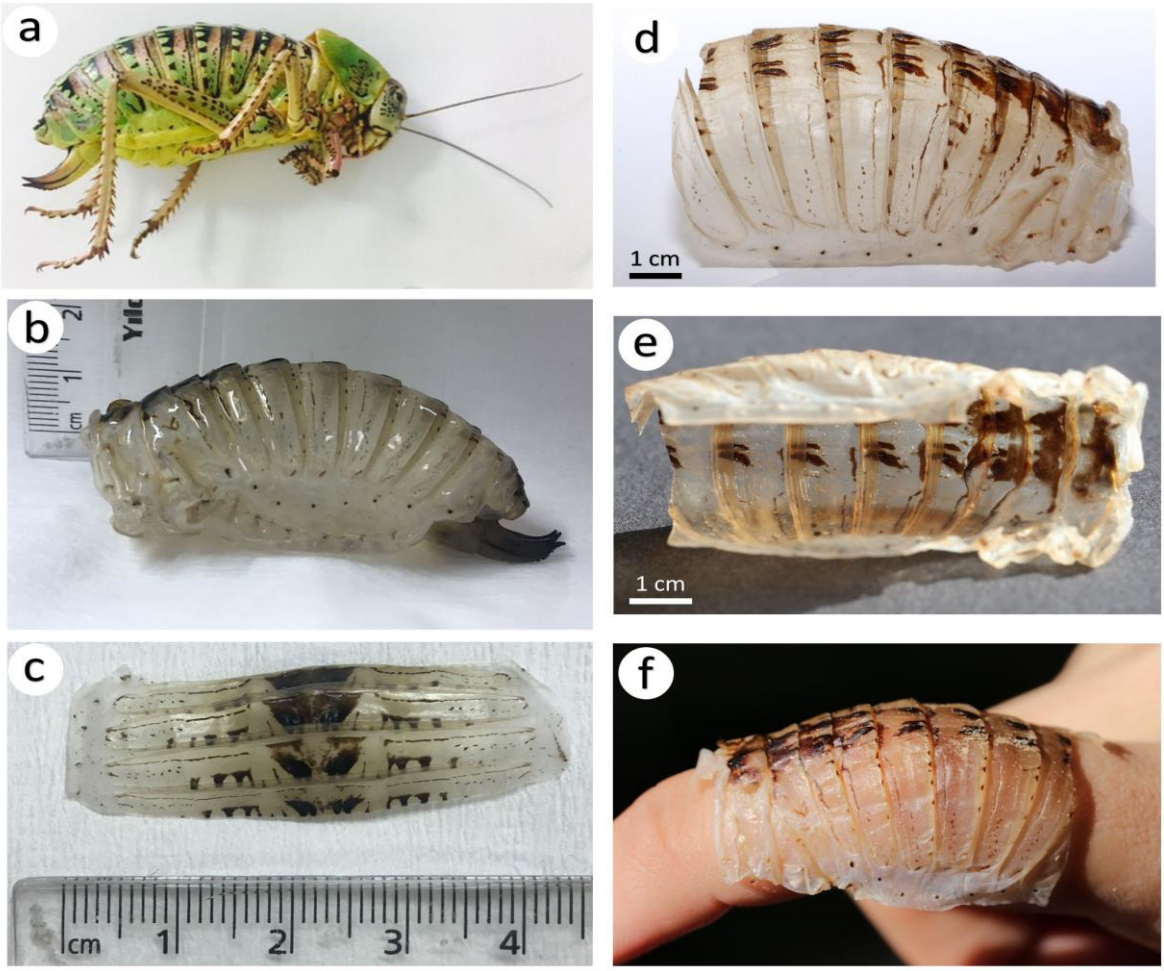

Figure 1. a) Bradyporus sp. lateral side. b) and c) Structure of 3D chitin produced from Bradyporus sp. d), e) and f) Structure of 3D chitosan produced from chitin

2.2. Chitin and Chitosan Isolation

All samples were demineralized in 1M HCl solution for 24 hours. The samples were washed with dH2O until the pH was neutral. It was then refluxed at 150°C for 6 hours using 1M NaOH solution. The deproteinization process was completed by washing the samples with dH2O to neutral pH and dried in an incubator at 60 °C for 4 hours. After incubating for 1 hour with a solution consisting of chloroform, methanol and dH2O (1:2:4) for decolourization, the samples were again washed with dH2O until pH neutral. Chitosan was obtained by deacetylation of chitin samples (Figures 1b and 1c), which were dried in an incubator at 50 °C for 48 hours, after adding 60% NaOH solution at 150 °C for 6 hours.

2.3. IR Analysis (FTIR)

FTIR of chitosan film samples (Figure 1d, 1e and 1f) were obtained with a Perkin-Elmer FTIR spectrometer over the frequency range of 4,000–625 cm^{-1}. The degree of acetylation (DA) of chitins was calculated as DA: [(A1658/A3450) x 100] /1.33 [32], The deacetylation degree (DD) of chitosans was calculated as DD (%):100- [A1658/A3450) x 115] [33,34].

2.4. Thermogravimetric Analysis (TGA)

The dynamic degradation studies for the film samples were carried out in EXSTAR S11 7300 thermal analyser simultaneous TGA/DTG (heating rate: 10°C min^{-1}).

2.5. X-ray Diffraction (XRD)

X-ray powder diffraction measurements were obtained at 40 kV, 30 mA, and 2θ with a scan angle from 5° to 45° using a Rigaku D max 2000 system. The crystalline indices (CrI) of chitosan isolated from Bradyporus sp. were calculated as CrI110 = [(I110− Iam)/I110] × 100 [34, 35]. (I110=maximum intensity at 2θ≅20°, Iam=intensity of amorphous diffraction at 2θ≅13°).

2.6. Scanning Electron Microscopy (SEM)

Gold plating of 3D chitosan film samples obtained without deteriorating their structure was done with Sputter Coater (Cressingto Auto 108). Surface morphologies were visualized with the QUANTA FEG 250 scanning electron microscope.

2.7. Elemental Analysis

The DS values of the amino group of the chitosan nanofibers were calculated from the C, S, N, O and H contents in the elemental analysis data by using the elemental analyzer.

2.8. Cell Culture

HEK293 (human embryo kidney) cells obtained from the American ATCC Cell Line Center were cultured in Dulbecco's Modified Eagle's medium (DMEM) culture medium containing 10% FBS, 1% Penicillin/Streptomycinsolution, 5% CO2 and in a 37°C incubator.

2.9. MTT Cytotoxicity Assay

The cytotoxicity of the chitosan was analysed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; M2128, Sigma) dye. After incubating cells (~1x104 cells) in 96-well plates for different times (24, 48, 72 hours) and at different concentrations (0-1000 and 1000-5000 µg/ml), the medium was removed. Incubation was continued at 37°C for 3-4 hours with 10 µL of MTT (5 mg/mL) dye. After adding 100 µl of DMSO (AppliChem, Germany) absorbance was measured at 570 nm in a CLARIOstar microplate reader. Cell viability percentage was calculated using the formula: [Cell viability %= (Atreated cells/Auntreated control cells) x 100] [36].

3. Results and Discussion

3.1. FTIR

FTIR has been used to characterize chitosan, similarly to Varma and Vasudevan, based on different bands corresponding to the $-NH_2$ group to concerning the symmetrical COO- collecting expanding vibration [37]. Chitosan FTIR spectra showed characteristic bands in the frequency range between 4000 and 400 cm^{-1}. While these bands appear as out-of-plane bends at values of 552.32 cm^{-1} (non-plane bending NH, out-of-plane bending C–O) and 892.29 cm^{-1} (out-of-plane bending NH); they showed themselves as sharp peaks at 1149.07 cm^{-1} (C–O–C bond), 2875.83 cm^{-1} (CH_2 bond) and 3361.29 cm^{-1} (−OH bond). Also, the vibrational mode of the amide C=O bond was determined at 1586.30 cm^{-1} (Figure 2a). In the literature, for chitosan; peaks of 1640 cm^{-1} (Amide I, C = O) (Chatterjee et al 2005), and 1590 cm^{-1} (Amide II, NH_2) (Peng et al 1994) were characteristically found.

3.2. TGA

The thermal stability of the chitosan film samples obtained from the body part of Bradyporus sp without deteriorating their three-dimensional structure by TGA was analyzed. The thermal endurance of the samples, as well as the water and ash content in the structure, were tested by increasing the temperature by 10 ˚C every 1 minute between the temperatures of 30˚C - 650 ˚C.

The mass loss in the first phase (between 30 and 73.3°C) indicates the evaporation of water. The water content of the chitosan membranes was determined as 4.7%. This loss is related to the evaporation of the total moisture content from the polymeric structure (Figure 2c).

The mass loss in the second phase (between 100 and 599 °C) indicates the degradation of carbohydrates and other molecules. Weight losses for chitosan in this degradation range were recorded as 56.3%. The intact fraction of chitosan (known as ash content) was recorded as 38.1%. Maximum decomposition temperatures (DTGmax) were determined as 300.5 °C for chitosan (Figure 2d).

DTGmax values of chitin and chitosan contents obtained from edible insects were 328.7°C for chitin, 314.7°C and 301.8°C for mealworm and commercial chitosan, respectively [38]. In previous studies, DTGmax values of chitin isolated from seven grasshopper species (Orthoptera species) be in the range of 381-385°C [2]. The DTGmax range of chitin obtained from six different aquatic invertebrates (Leptinotarsa decemlineata species) is (350-393°C), while the DTGmax range of chitosan is (208-308°C) [34].

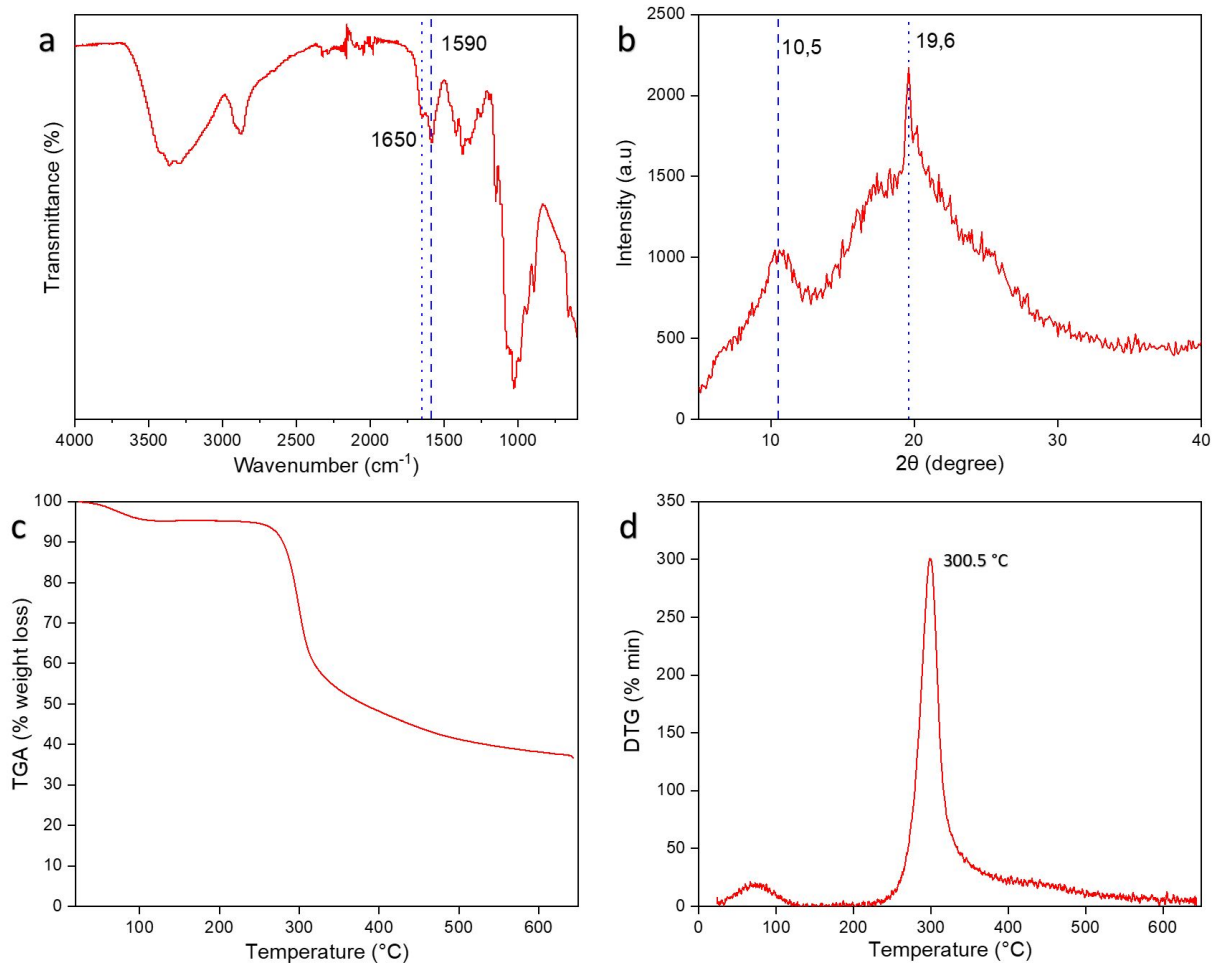

Figure 2. a. The FTIR spectra of chitosan prepared from Bradyporus sp. b. Graph of intensity/2θ (°) plotted with data after XRD diffraction analysis. c. The TGA spectra of chitosan prepared from Bradyporus sp. d. Graph of DTG (%min)/Temperature (°C) plotted with data DTG analysis.

3.3. XRD

Based on the unique atomic sequences of the chitosan samples, XRD analysis was performed. With this analysis, the peak values of chitosan samples produced from plump grasshoppers were determined. As a result of XRD analysis of chitosan, two peaks were detected in the ranges of 2θ =10.2-10.9 and 2θ=19.5-19.7 (Figure 2b). On the XRD graph, the first peak is weaker than the second peak. These peaks reached the maximum at 2θ=10.9 and 2θ=19.6. Looking at the literature, many XRD models of chitosan have two characteristic peaks, usually around 2θ=10 and 2θ=20 [39]. The XRD results obtained in our study are in the range of the literature information.

3.4. SEM

The surface morphologies of chitin and chitosan contents obtained from different organisms may differ. According to the SEM images, the surface morphologies may be only in microfibrillar structure, as well as in microfibrillar structure and porous/non-porous form [34]. The surface morphologies of the chitosan film samples obtained in our study were examined in detail by SEM analysis at different magnifications (5000-40000X). In some areas, rough, irregular membrane cracks, and irregularly patterned, non-porous and smooth surfaces without chitosan nanoparticles were also detected. On the other hand, irregular microstructure and the presence of very small pores are also observed in some regions (at high magnification). Inhomogeneous surface morphologies can be detected as the obtained chitosan content is taken from the body region of Bradyporus sp. (Figure 3).

3.5. Elemental Analysis

Elemental analysis was used to determine the elemental percentages of carbon (C), sulfur (S), nitrogen (N), and hydrogen (H) in the structure of the obtained chitosan with high precision. While no sulfur was found in the content of chitosan, ~41.10% carbon, ~7.46% nitrogen and ~6.67% hydrogen were detected (Table 1). The ~7.46% nitrogen in chitosan content is consistent with the literature [40], and is found more than chitin.

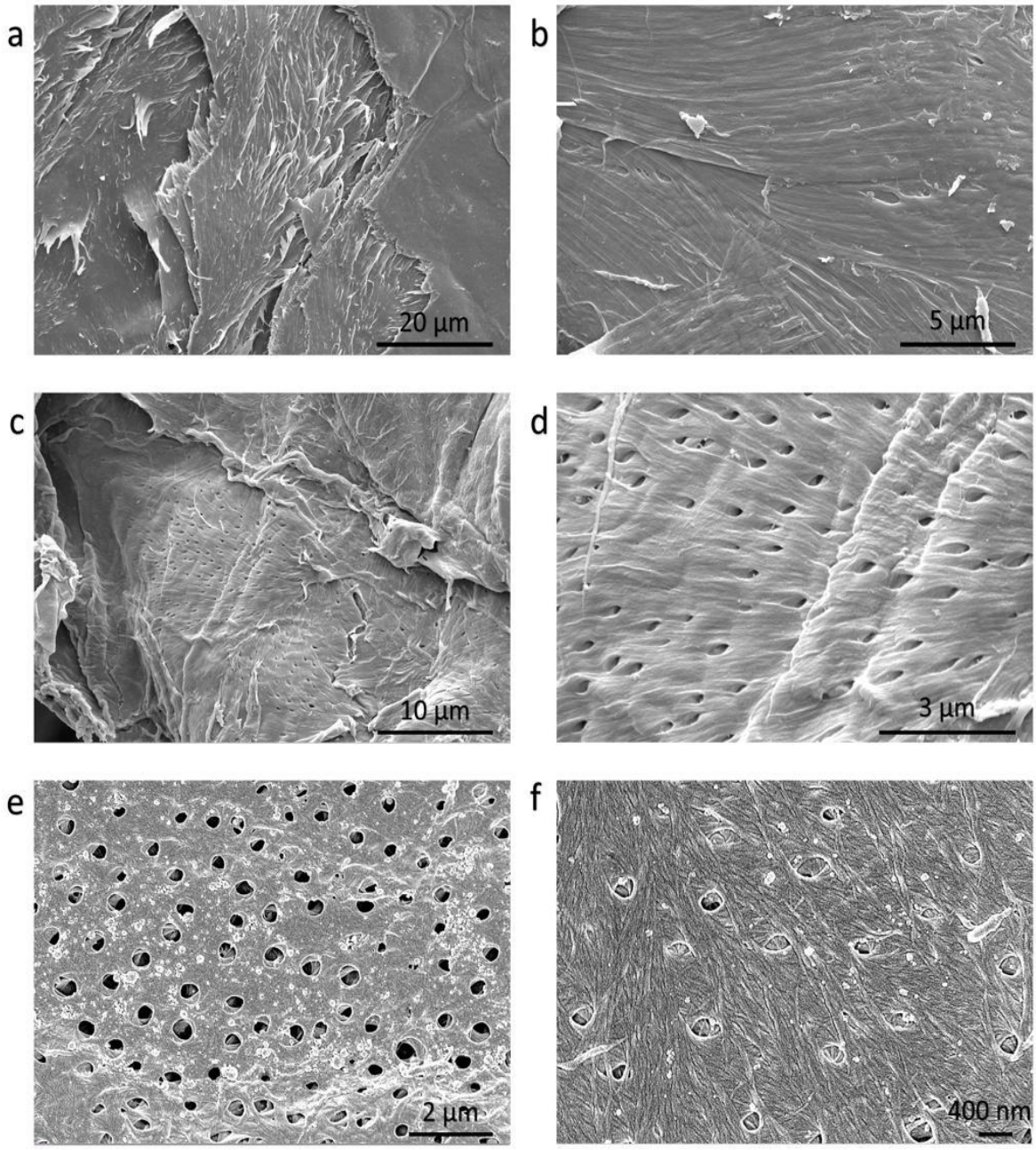

Figure 3. Images obtained as a result of SEM analysis. a. The image was obtained with 5000X magnification power, b. Images obtained with 20000X magnification power, c. Images obtained with 20.000X magnification power, d. Images obtained with 40000X magnification power, e. Images obtained with 40000X magnification power, f. Images obtained with 40000X magnification power.

Tablo 1. Chitosan elemental analysis results.

Polymer	%N	%C	%H	%S
Chitosan	7.469594479	41.10246277	6.670635223	0

3.6. MTT Cytotoxicity Assay

To determine the cytotoxic effect of chitosan, whose characterization has been completed, on HEK293 cells, an MTT test was performed at different concentrations in the range of optimal dose (0-1000 µg/ml) and overdose (1000-5000 µg/ml) (Figure 4). While no inhibition of IC50 was observed at 24 hours in the optimal dose range (Figure 4d), an initial IC50 value of 1000-2000 µg/ml was detected in the overdose range (1427 µM, Figure 4g). IC50 values were determined at the optimal dose intervals (800-1000 µg/ml, 808.9 µM) and (600-800 µg/ml, 611.6 µM) at the 48th and 72nd hours, respectively (Figure 4h and Figure 4i).

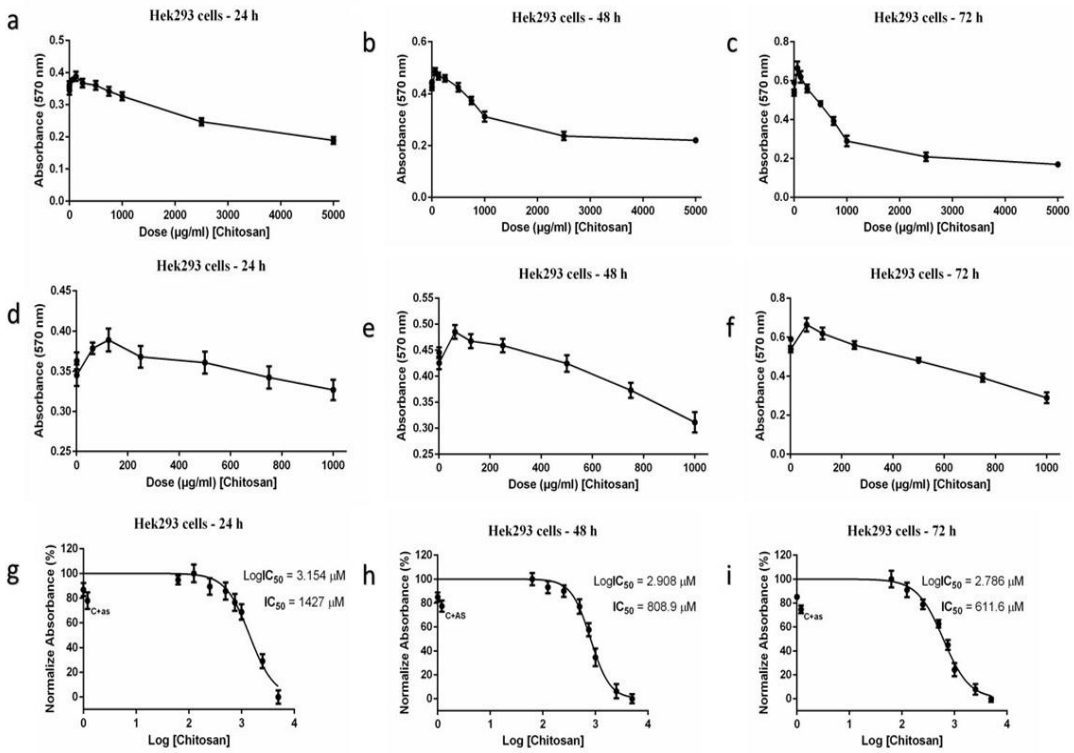

Figure 4. Absorbance (570 nm) and normalize absorbance (%) graph of HEK-293 cells. a., b., c.) overdose range; d., e., f.) optimal dose range; g., h., i.) IC50 values at 0-5000 µg/ml dose range.

4. Conclusion

Insects are a good source of chitin and chitosan due to their abundance in nature and easy culturability. However, the chitin and chitosan contents of each insect species are quite different from each other. Chitosan was detected in the plump grasshopper species discussed in this article and characterized by different methods. The obtained chitosan did not show cytotoxic effects at optimal doses at 24 hours. From this point of view, it is possible to say that the obtained chitosan polymer is non-cytotoxic up to the overdose range, is biocompatible and has the potential to be used in the biomedical field. This plump grasshopper species, whose chitosan structures are characterized, can be produced in a controlled manner and used as a potential source of chitin and chitosan for various technological and industrial purposes.

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