Characterization and Biocompatibility Evaluation of Chitin Film Produced from Head of Caligula japonica Pupa: From Waste to Use

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Research Article

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Abstract

Harmful insects become bio-waste after they are collected for plant protection purposes. It is very important industrially to transform these bio-wastes into technological materials rather than incineration. *Caligula japonica* damages many plants such as walnut, chestnut, and apple, reduces fruit yield and even causes the trees to die. In this study, a three-dimensional natural chitin film was produced from the head of the *C. japonica* pupa, which is collected in large quantities from nature. As a result of the FTIR analysis, it was revealed that the produced films were in the form of alpha chitin and consisted of nanofibers that could be observed very clearly according to SEM analysis. The maximum degradation temperature of the resulting chitin film was measured to be 367.8 °C. As a result of XRD analysis, two sharp peaks were recorded in 9.42 and 19.52 which are characteristic for chitin. The cytotoxicity analysis performed on L929 revealed that the chitin film did not have any cytotoxic effect at 24th, 48th and 72nd hours. As a result, a natural, three-dimensional, nanofiber film with biocompatible properties was produced to be used in future biomedical applications.

1. Introduction

Chitin is a β-1,4-linked N-acetyl D-glucosamine polymer which is linear in glycosidic junction geometry [1]. Thanks to its strong antimicrobial, antioxidant activity, non-toxicity and biodegradability properties, this polymer has a wide range of application areas such as medicine, pharmacy, tissue engineering, biotechnology, biomedical and food industries [2]. Chitin is primarily found in the cell wall of fungi and also known to be found in the structure of more than 70% of living things in Arthropoda, Bryozoa, Tardigrade and Mollusca phylums. In many studies in the literature, chitin was reported to be isolated from outer shells of crabs, shrimps and lobsters; from the cell wall of fungi and some algae; and from the whole-body structure of some species such as corals, sponges and insects [1, 3, 4]. In this study, the head structure of the silkworm pupa was used as the source of chitin.

Caligula japonica (*C. japonica*), previously called as *Dictyoploca japonica*, *Saturnia japonica* and *Dictypoea japonica*, belongs to Caligula, Lepidoptera: Saturniidae genus [5, 6]. This native insect pest is widely distributed in China as well as Japan, North Korea, and Russia [7, 8]. *C. japonica* has a wide range of 38 host plants from 30 genera and 20 families. Host plants include walnuts [9, 10] and *C. japonica* larvae mostly feed on walnut leaves. The infestation of this pest locally causes serious problems and economic loss in agricultural sector, and there is a common concern about further spread [8, 11]. Although chemical pesticides are currently the most common method used to keep this pest under control, due to its limitations, the search for safer and more effective methods continues.

Chen et al. (2021) obtained natural chitin film from the pupal shell of these insects and determined the surface morphology by performing detailed characterization of these films in their studies. They also studied the SERS activity for both surfaces (dorsal and ventral) of the films and predicted that this structure would contribute to materials science. However, no studies were carried out where 3D chitin film was produced from the head of *C. japonica* pupa.

In this study, three-dimensional chitin film was produced from the head of *C. japonica* pupa for the first time, and it was characterized by FT-IR, TGA, XRD, elemental analysis and SEM analyses from a biomaterial point of view. The biocompatibility of the three-dimensional chitin film produced, was tested in L929 mouse fibroblast cells by MTT method. This helped determining whether the produced film is pure chitin, clearly revealing its crystal structure, thermal stability, surface morphology and evaluating its biocompatibility [12].

2. Material And Methods

2.1. Sample collection and materials

Some parts of Chen et al. (2021) work were used in current study. The samples were stored as dry material in isolation, without any exposure to heat or light until chitin isolation. All chemicals used during chitin isolation were obtained from Sigma Aldrich. Distilled water was used throughout the study [12].

2.2. Chitin Isolation

The samples were washed with distilled water in order to remove possible impurities before chitin extraction, and then dried in an oven at 50°C for 3–4 days. Demineralization of *C. japonica* pupa head samples was performed with a 2 M HCl (50 ml) solution at 50°C for 6 hours using a magnetic stirrer. After the acid treatment, samples were washed with distilled water until they reached neutral pH and then dried in an oven at 50°C for 3 days. To remove the protein in their structure, dried samples were transferred to 2M NaOH (50 ml) solution and mixed with a magnetic stirrer for 8 hours at 100°C. Afterwards, the samples were washed with distilled water until they reached neutral pH. At the end of the isolation, 3D shape of *C. japonica* pupa head was preserved and a natural chitin film was produced. Finally, the samples were dried at room temperature for one week for analysis. Initial weights of *C. japonica* pupa head samples were determined before isolation. Following the isolation stages, samples were weighted again, and the content of chitin was determined using the following formula.

Chitin content (%) = $(m_{last}/m_{first}) \times 100$

m_{last}: mass of pure chitin after isolation; m_{first}: mass of sample before isolation

2.3. Characterization Studies

2.3.1. Thickness and Appearance of the Films

The images of the chitin film isolated from the head structure of *C. japonica* pupa were taken using a light microscope (Leica Z6 APO). The thickness of the films was measured using a digital micrometer

Mitutoyo (Coolant Proof Micrometer-293). The thickness was calculated as the average of measurements taken from 10 different pieces.

2.3.2. Fourier Transform Infrared Spectroscopy (Ft-ir)

Infrared spectra of *C. japonica* pupa head chitin isolates were recorded at 4000 – 600 cm^{-1} range and 64 scans, 8 cm^{-1} resolution using Bruker/Vertex 70 HYPERION Spectrometer.

2.3.3. Thermogravimetric Analysis (Tga)

The thermal stability, water and ash content of chitin samples isolated from the head structure of *C. japonica* pupa were analyzed (TGA Exstar-TG / DTA 7300 Instruments). Samples were tested by heating at a constant temperature of 10 ˚C min^{-1} from 30˚C to 700 ˚C under nitrogen atmosphere, using a platinum crucible.

2.3.4. X-ray Diffraction (Xrd)

The operations of this analysis which is based on refraction of X-rays in a unique order depending on the characteristic atomic arrangement of samples, were carried out with Panalytical/ Empyrean between 5–45˚ at 45 kV and 30 mA (Bruker AXS D8 Advance). The % crystallinity was determined by using the intensities of the peaks obtained as a result of XRD analysis. The following formula was used when calculating the crystallinity:

$CrI110 = [(I110 - Iam) / I110] \times 100$

CrI = % crystallinity value,

I110 = maximum intensity value at 2θ 20°,

Iam = Maximum intensity value of the amorphous peak at 2θ 13°

2.3.5. Elemental Analysis

This analysis was carried out with Thermo Flash 2000 to determine the percentages of C, N, O and H elements in the structure of the samples with high precision. The DA value of the samples to the determined % element contents was calculated with the following formula:

$$DA = [(C/N-5.14)/1.72] * 100$$

where C:N is ratio of carbon to nitrogen (w/w).

2.3.6. Scanning Electron Microscope (Sem)

The surface morphologies of the chitin samples were defined by SEM analysis in various magnifications at 5 kV and between the range of 500X-30.000X (JEOL JSM-6335F). Samples were coated with gold/palladium before the analysis (JEOL JFC-2300 HR).

2.4. Cell Culture Studies

2.4.1. Growth of Cells

L929 (Mouse fibroblast, HUKUK, Sap Institute) cells were used to determine the biocompatibility of the chitin film sample, isolated from the *C. japonica* pupa head material. Cells were grown in incubator as monolayer cultures in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 units/ml) and streptomycin (100 µg/ml) at 37ºC and 5% CO2 at 95% humidity. Cells were checked on a daily basis with an inverted microscope, media were changed every other day, and passaged when 80% confluent.

2.4.2. Cytotoxicity Analysis

For MTT analysis, confluent cells at the base of the flask were removed from the flasks using trypsin-EDTA solution and counted on the Thoma slide after being stained with 0.4% trypan blue. Experimental studies started when the cell viability rate was 90% or higher. Cells were seeded as 1×10^4 cells per well (96 wells) and culture plates were incubated for 24 hours at 37°C in an incubator humidified in 5% CO_2. After 24 hours, sterilized (with 70% Ethanol and UV combination) biomaterial with a diameter of 2 mm were added to each well, and the cultures were retained in the incubator with previously specified conditions, during the incubation periods (24, 48 and 72 hours) without any medium changes. Untreated (biomaterial free) control cells were also retained to be used for growth inhibition comparison. The cytotoxicity of the chitin structure, isolated from the *C. japonica* pupa head material, on cells was evaluated using the conventional MTT test. The biomaterial in the wells were removed after the incubation period and MTT solution (0.5 mg mL^{-1}) was added to all application and control wells, and the plates were incubated in the dark at 37°C for 3 hours. Then the lysate was removed and DMSO was added to the wells to dissolve the formazan crystals. After waiting 5 minutes for the stabilization of the color, it was read with a ChroMate®ELISA reader at 492 nm. The viability of biomaterial free untreated cells was considered as 100%. Cell viability percentage (%) was calculated based on the following parameter.

$$\% \text{ Viable cells} = ((A_{sample} - A_{blank}) / (A_{control} - A_{blank})) \times 100$$

$A_{control}$ is absorbance of the control, A_{sample} is absorbance of wells with biomaterial added, and A_{blank} is absorbance of the medium without cell.

2.4.3. Statistical Analysis

Data analysis was performed using GraphPad Prism software version 8 (GraphPad Software®). Data represent mean – standard error of the mean (SEM). Statistical differences were assessed by Two-way ANOVA followed by Bonferroni post-test. * was regarded as statistically difference ($p < 0.05$).

3. Results And Discussion

3.1. Chitin content, appearance and thickness

The chitin content of the film, isolated from the head structure of *C. japonica* pupa was determined as 49.06%. The thickness of the chitin film was measured as 0.120 ± 0.0034 mm. Surface morphology was determined by light microscopy (Supp file 1).

3.2. FT-IR

The FT-IR spectrum of the chitin isolated from the head structure of *C. japonica* pupa is given in Fig. 1. There are two major peaks in the FTIR analysis of chitin, that are Amide I and Amide II. According to the literature, two sharp peaks split around 1660 and 1620 cm^{-1} indicate the α-form of chitin, and an undivided peak around 1640 cm^{-1} indicate the β-form of chitin [13–16]. It is clearly observed that the Amide I band is split into two in the FTIR spectrum of the chitin isolate obtained from the head structure of *C. japonica* pupa. Amide I peak appears significantly at 1654.48 and 1619.49 cm^{-1} in the spectrum of chitin isolate, consistent with the literature. Another major peak in the characterization of the chitin is Amide II band. This peak is around 1552 cm^{-1} for pure chitin. The Amide II band recorded for the chitin isolate obtained from the head structure of *C. japonica* pupa is 1552.00 cm^{-1}. Amide II band was also consistent with the literature. The peak observed at 1306 cm^{-1} is attributed to Amide III band [17]. The relatively wide peak of 3434 cm^{-1} recorded in the spectrum is attributed to O-H stretching [18, 19]. All these peaks in the recorded spectrum support the purity of the chitin isolated from the head of *C. japonica* pupa. Results of the FTIR analysis of the structure are given in Fig. 1. Comparison of relevant material with commercial chitin and whole body of *C. japonica* is given in Supp. File 2.

3.3. TGA

In this study, the thermograms of the TGA analysis performed to reveal the temperature deterioration of the chitin samples obtained from the head structure of *C. japonica* pupa are given in Fig. 2. Differential thermogravimetry (DTG) thermograms were produced as the heating rate increased. Mass loss in 0-100°C range is due to the removal of intermolecular water of the biopolymer [13]. In this range, a very slight peak was observed in the thermogram of chitin samples obtained from the head structure of *C. japonica* pupa. The peak recorded in the second step is known to result from the degradation of the chitin structure and acetylated chitin units, and also from the dehydration of the saccharide ring [20, 21]. The mass loss at

this stage was determined as 65%. It has been noted in the literature that, chitins isolated from different organisms lost mass in two stages, as in the current study [13, 14, 21–25].

These results support the success of chitin production from the head structure of *C. japonica* pupa. In the literature, maximum decomposition temperature of α-chitin is reported to be above 350°C [21, 26]. In the current study, the DTGmax (°C) value of the chitin sample from head of *C. japonica* is 367.8°C. The recorded value is consistent with the literature. Chitin stability increases in direct proportion to thermal decomposition temperature, and thermal stability is an important parameter to consider when choosing the application area for a biopolymer such as chitin [27].

3.4. Elemental Analysis

The elemental analysis results and degree of acetylation (DA) of chitin obtained from the head of *C. japonica* pupa are given in Table 1. The N, C, and H content of chitin isolates were 6.74%, 48.23%, and 6.84% respectively. Theoretical percentage of N for pure chitin is stated as 6.89% in the literature [24]. The recorded N value for the chitin obtained from the head structure of *C. japonica* pupa is quite compatible with the literature. Considering the elemental analysis data, the degree of acetylation (%DA) of the thin chitin film was determined as 87.29%. The obtained degree of acetylation depends on factors such as purification process, analytical methods, and sources of chitin [28].The degree of acetylation and N content, reveal the purity of the isolated chitin content. Degree of acetylation values were found to be compatible with previous chitin studies [24, 29, 30]. The results are given in Table 1.

Table 1
Elemental analysis and degree of acetylation (DA) of chitin, obtained from the head structure of *C. japonica* pupa

Chitin sample	%N	%C	%H	% DA
Head of *C. japonica* pupa	6.74	48.23	6.84	87.29

3.5. X-ray Diffraction (Xrd)

XRD was used to distinguish three different allomorphic forms (α-, β- and γ-chitin) of chitin molecules in the head structure of *C. japonica* pupa. According to the literature, there are characteristic peaks in XRD analysis of pure chitin. These are two major peaks around 9° and 19° and two fair, minor peaks around 21°, 23° [31].

The XRD analysis results of the chitin in the head structure of *C. japonica* pupa are shown in Fig. 3. Major peaks in XRD diffraction of the isolated, thin chitin film were recorded at 9.42° and 19.52°. These peaks

are fully consistent with the literature and support the purity of the isolated chitin [24]. CrI values of the obtained chitin were %79.73 when calculated according to the formula.

3.6. SEM

SEM images of both dorsal and ventral parts of the chitin obtained from the head of the *C. japonica* pupa are shown in Figure X. While the dorsal surface of the isolated chitin film consisted of nanofibers (Fig. 4a, b, c), rough structures were observed on the ventral surface (Fig. 4d, e, f). As is known well, the nanofibers are characteristic for the chitin ; however the rough chitin surface also reported in the literature [4]. Here in the present work, it is gained to literature that natural chitin film may have double surface characteristics at nanoscale. This observed variations on both sides of the chitin film can be used for materials applications in further studies.

4. Cell Culture Studies

4.1. Cytotoxicity Analysis

MTT test was performed to evaluate the cytotoxic activity of the chitin film isolated from the head structure of *C. japonica* pupa. The colorimetric method was used to evaluate the cytotoxicity of the film on L929 cell line and to determine the percentage of viable cells. The MTT test is based on the conversion of tetrazolium salts into water-insoluble formazan crystals. The formazan crystal is dissolved after adding DMSO and the resulting color is measured with an ELISA reader. Absorbance is directly proportional to the number of viable cells.

As a result of the MTT test, cell viability was recorded as 100% and 90.68% in the control groups without film and application groups containing film, respectively within 24 hours. Viability percentages at 48th and 72nd hours were determined as 84.86% and 82.08%, respectively. According to cell viability results, it was concluded that the chitin isolated from the head structure of *C. japonica* pupa with a diameter of 2 mm, did not have a cytotoxic effect on L929 cells at the indicated hours (viability percentages of L929 cells were over 70% in all three time periods). According to the statistical evaluation, only the change at 72nd hour was significant ($p < 0.05$) (Fig. 5).

5. Conclusion

Chitin was successfully isolated from the head structure of *C. japonica* pupa. As a result of the FTIR analysis, it was determined that the isolated chitin was in the α form, the peaks were consistent with the literature, and the sharpness of these peaks showed that the chitin was isolated in a pure form. The chitin, isolated from the head structure, showed high thermal stability. TGA analysis supported that the chitin was in α form. The N content determined by elemental analysis and calculated DA value revealed the purity of the chitin. It was also supported by XRD analysis that the chitin was in α form and isolated in pure form. SEM analysis revealed that the isolated chitin was consisted of fibers and pores. According to

the cytotoxicity analysis, it was determined that the material was not toxic to cells and was biocompatible. Considering these advantages, it is predicted that it can be used effectively in the medical field.

Declarations

Statement Novelty

In this study, chitin film was isolated from the pupa head of *C. japonica* for the first time, and the biomaterial was characterized using FT-IR, TGA, XRD, elemental analysis, and SEM methods, and the biocompatibility of the material was tested by the MTT method in L929 mouse fibroblast cells. In this way, whether the isolated structure is pure chitin, its crystal structure, thermal stability, and surface morphology were revealed and its biocompatibility was determined.

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Figures

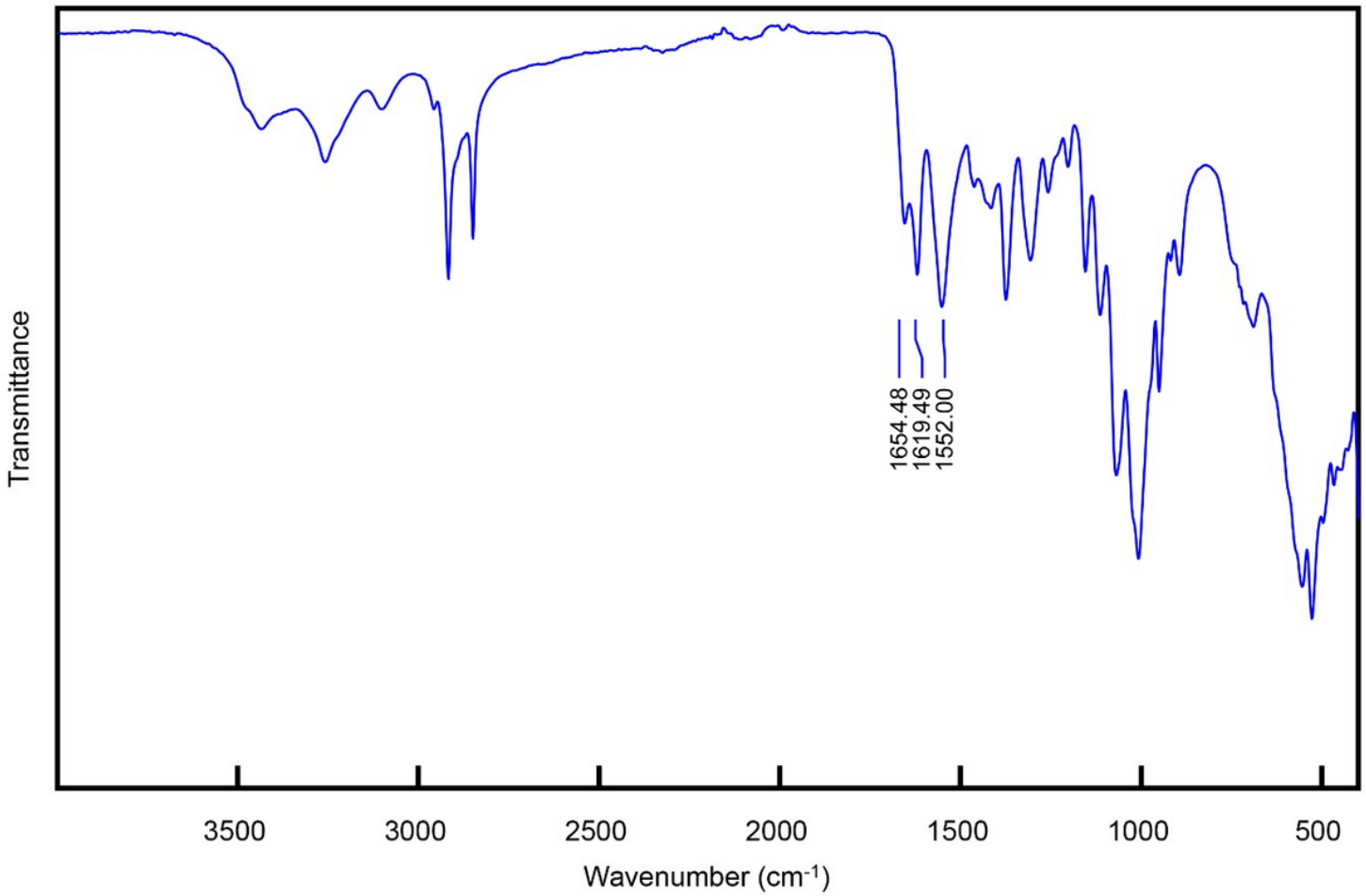

Figure 1

FTIR spectrum of the chitin obtained from the head structure of *C. japonica* pupa.

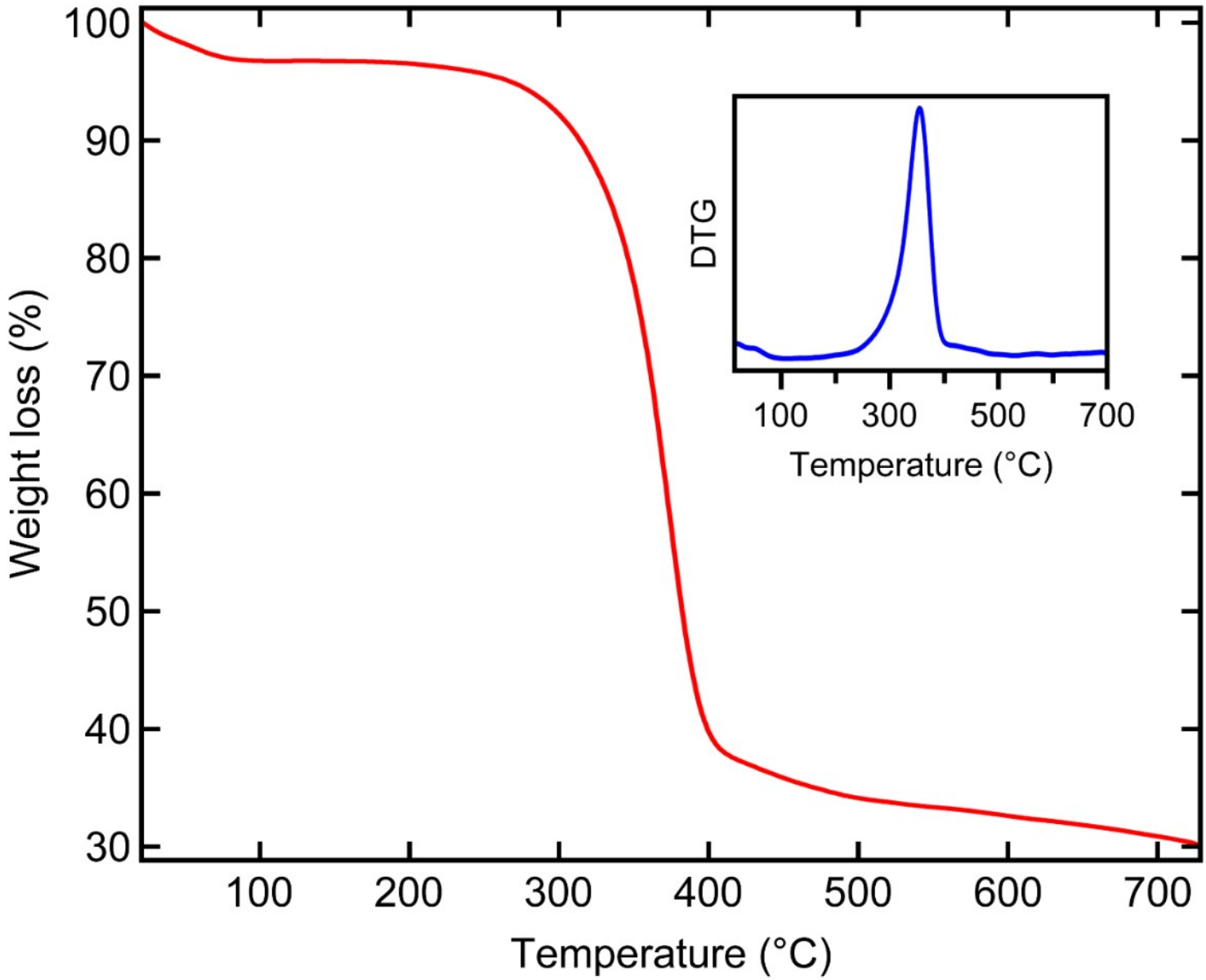

Figure 2

TGA thermogram of the chitin samples obtained from the head structure of

C. japonica pupa.

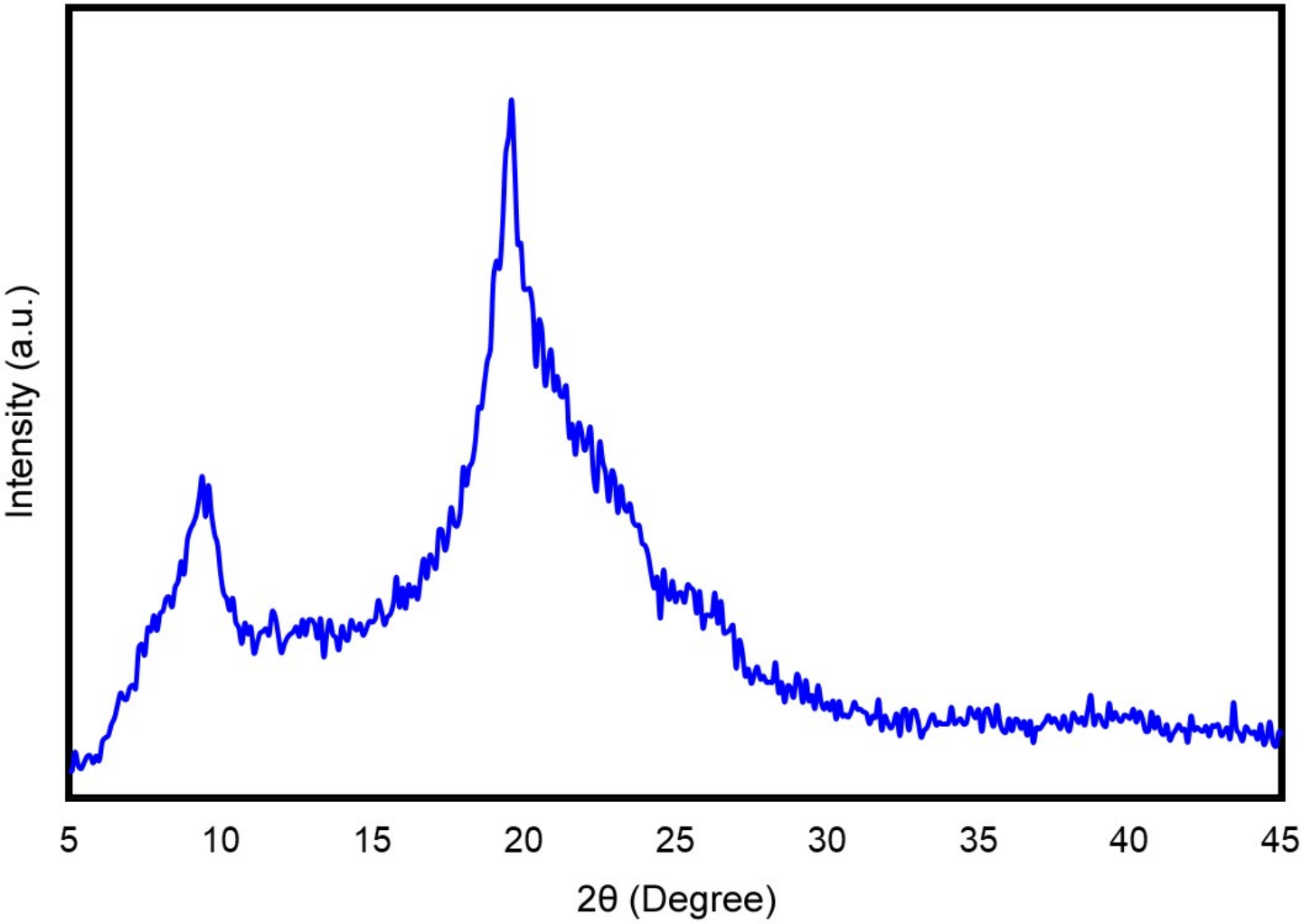

Figure 3

X-Ray diffraction curve of the chitin samples obtained from the head structure of *C. japonica* pupa.

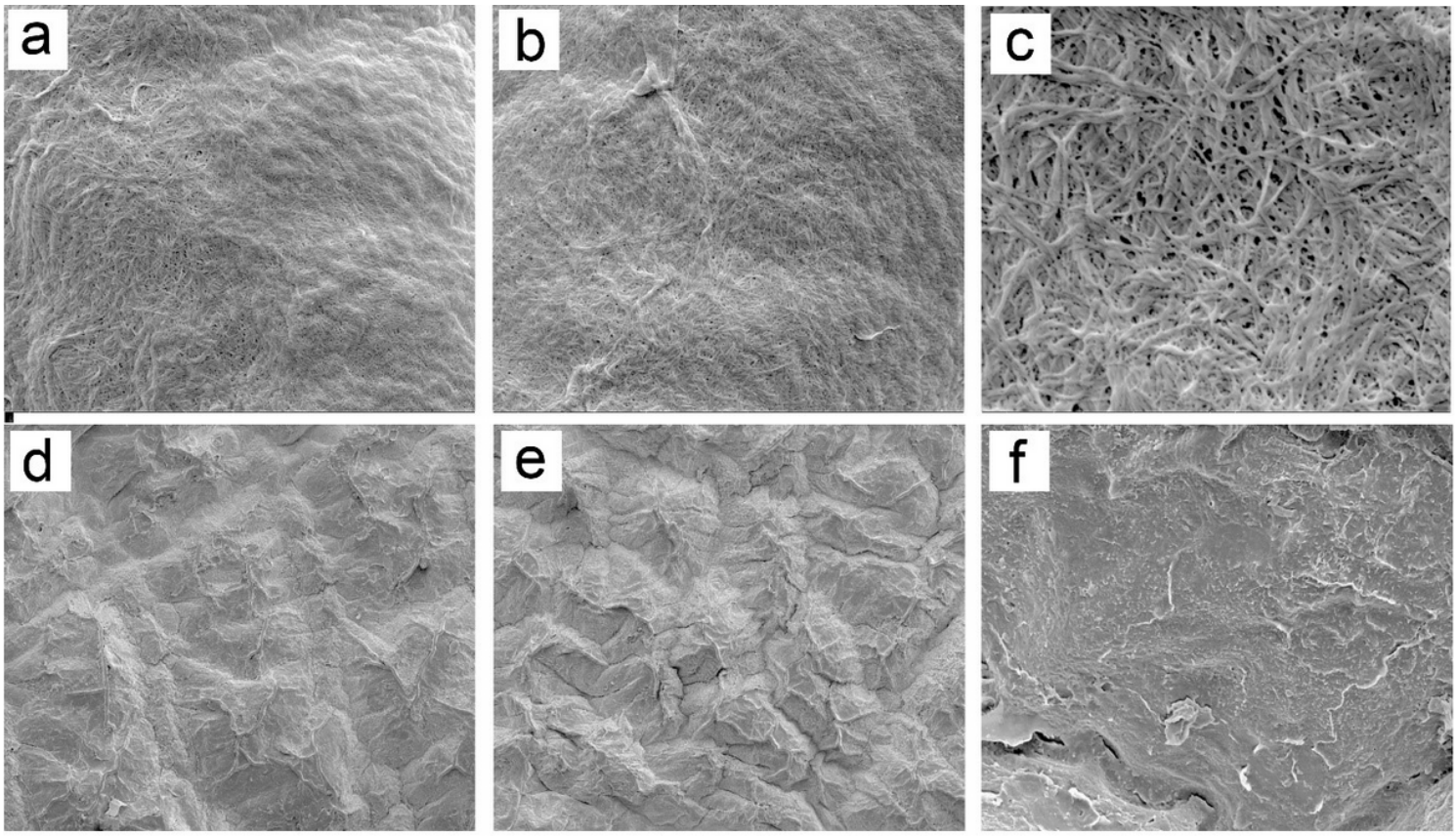

Figure 4

SEM images. a, b, c) dorsal side views of the chitin obtained from the head of the *C. japonica* pupa, d, e, f) ventral side views of the chitin obtained from the head of the *C. japonica* pupa.

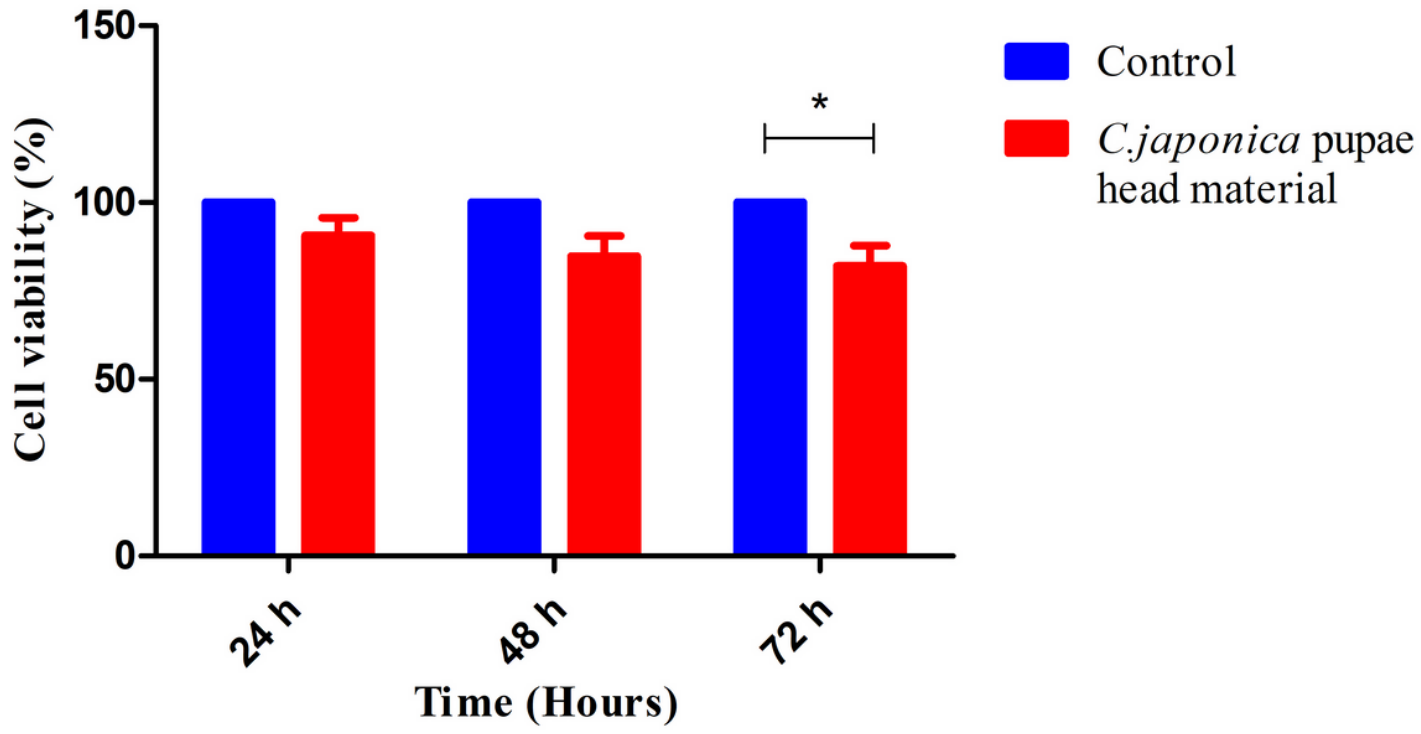

Figure 5

Cell viability assays of L929 cells after cultured on *C. japonica* pupa head material for 24, 48 and 72 hours (Bar represent mean of cell viability ± SEM; n=3 statistical difference is showed as * p< 0.05).

Supplementary Files

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