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In-vivo Safety Evaluation of Chitosan Extracted from the Pupal Exuviae of *Hippotion celerio* L.

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ABSTRACT

Chitosan, an important and abundant natural-based polymer, was extracted for the first time from the pupal exuviae (PE) of *Hippotion celerio* L., reared and fed with an invasive plant species, *Colocasia esculenta* (L.) Schott. In this study, we investigated the safety and biotechnological potential of insect-based chitosan. Demineralization and deproteinization were performed with 1 M hydrochloric acid at 25°C for 3 hours at 300 RPM and 1 M sodium hydroxide (NaOH) for 4 hours at 100°C, respectively. The extracted chitin was converted into chitosan via deacetylation using 50% NaOH for 4 hours at 80°C. Both chitin and chitosan were characterized by Fourier Transform Infrared Spectroscopy (FTIR) to determine its purity and Scanning Electron Microscopy (SEM) to examine its surface morphology. For the acute toxicity study, a single dose of three concentrations of 300, 2000, and 5000 mg/kg bw of chitosan were administered orally using a gavage to healthy female Swiss albino mice. The chitin and chitosan yield of the dry weight were 21.74±2.15% and 58.26±4.24%, respectively. Additionally, the degree of deacetylation (DD) was found to be 85%, higher compared to the commercial chitosan obtained from the shrimp with 83%. Scanning Electron Microscope (SEM) exposed smooth, porous, and fibrous surfaces, suggesting its use in biotechnology. LD_{50} value for chitosan in Swiss albino mice was greater than 5000 mg/kg bw. Thus, this study suggests that chitosan extracted from the pupal exuviae of *H. celerio* L. is safe and could be used in future applications in tissue engineering, biomedicine, and textile industry.

***Keywords*:** Fourier Transform Infrared Spectroscopy, Scanning Electron Microscopy, Deacetylation, Acute toxicity, Swiss albino mice.

Introduction

Insect species, such as those belonging to Family Sphingidae, are known to have an exoskeleton (pupal exuviae), which is primarily made up of chitin, a natural biopolymer widely spread on Earth secondary only to cellulose.[1,2] This polymer when deacetylated into chitosan ((1→4)-2-amino-2-deoxy-*β*-d-glucan) has several potential applications.[3] It was reported to have antioxidant, hypocholesterolemic, immune-stimulating, antitumor, antimicrobial properties, and anti-inflammatory activity.[4,5,6] Chitin and chitosan are categorized as biodegradable, biocompatible, and non-toxic substances for human use.[7,8] They are primarily derived from crustacean's waste. However, chitosan from marine sources has drawbacks, such as allergic components that are harmful to human health,[2] environmental pollution and crustacean shell disease hampering the collection of these polymers as well.[9] Chitin and chitosan from insect species are a potential alternative source due to their practical applications and higher output compared to shellfish.[1,10] They can be raised with fewer resources, produce more output and have lower emissions of greenhouse gases and pollutants.[11,12,13] According to the previous study of Suelo *et al*, the life cycle of *Hippotion celerio* L. (Silver-striped hawkmoth) at the emergence of an adult range only to 32-34 days, where pupal exuviae can already be collected.[14]

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This is quite comparable to the crustacean species as the main source of chitin, which could take up to 75 days to mature.[15] Chitin is the major component of pupal exuviae.[16] Deproteinization, demineralization and depigmentation are the three vital steps needed to produce a chitin.[17] The physicochemical properties of chitin and chitosan were characterized by Fourier Transform Infrared Spectroscopy (FTIR) and Scanning Electron Microscopy (SEM) in order to validate its purity and to examine the surface morphology of the extracted polymer, respectively.[18] Despite numerous reports on the composition and characteristics of different insect-derived chitins and chitosan, precise isolation and characterization of these are still lacking, and insects that are members of the Sphingidae family are yet to be investigated in order to maximize the biopolymer.[19,20] In this study, the chitin and chitosan previously isolated from the pupal exuviae of *H. celerio* L. hawkmoth species were characterized and selected based on pilot scale studies to explore its safety using animal models following the Organization for Economic Cooperation and Development (OECD) no. 423 guidelines.[46]

Materials and Methods

Chemicals

Reagent such as hydrochloric acid, acetic acid and sodium hypochlorite were purchased from Joelmar Trading (Iligan, Philippines) while sodium hydroxide was procured from HiMedia (Maharashtra, India). All solvents used were of analytical grade. Commercial chitin and chitosan (degree of deacetylation: >=75%), derived from shrimp shells, were purchased from Sigma-Aldrich (St. Louis, Missouri, USA) and HiMedia (Maharashtra, India), respectively.

Ethical Statement
All experiments were conducted in accordance with the Institutional Animal Care and Use Committee's (IACUC) regulations, and all effort was made to limit the animals' suffering. For the use of animals in the study, a letter of approval from the IACUC (Protocol Number 2024-420D) was secured prior to the conduct of the study.

Acquisition of Permits
Wildlife Gratuitous Permit (WGP: Michelle Suelo No. R10 2024-06) was secured per DENR AO 2004-55 for the collection of hawkmoth species (*Hippotion celerio* L.) from Musuan, Bukidnon, Philippines (7°52'53.88463" N 125°3'49.96444" E, 9 April 2024). Appropriate laboratory permits were also secured.

Rearing of Hippotion celerio L.
The collected *H. celerio* L. adults from the light trapping technique were reared until they began to lay eggs. Eggs were then collected, and when they turned into their first instar, they were fed using its host plant, *Colocasia esculenta* (L.) (Taro), an invasive plant species, until they reached their last instar, turning them into pupa.[21,22] Once the pupa turned into an adult, the pupal exuviae were collected, air-dried and stored as a vacuum-dried material without further exposure to heat, humidity or light up prior to the conduct of chitin extraction.

Preparation of Chitin
The extraction of chitin from the pupal exuviae was carried out based on the process reported by Pourebrahim *et al.* [2] with minor modifications on the pretreatment of pupal exuviae as well as on the temperature and the number of revolutions per minute (RPM) during the purification step. This was done in three replicates. Prior to chitin extraction, the collected pupal exuviae samples were washed several times with distilled water to remove any impurities present on the specimens and dried in an oven (Memmert U75) at 50°C for 48 hrs. All dried samples were partially ground using a portable electric grinder. To extract the minerals from the pupal exuviae, the samples were suspended in 1 M HCl solution at 25°C for three (3) hours at 300 RPM using a magnetic stirrer (Corning PC-420D). After HCl treatment, all samples were filtered first using a Whatman filter paper (400 x 400 mm), then by cheesecloth and washed with distilled water until a neutral pH (pH7) was obtained. Proteins were removed from demineralized samples by treatment with 1 M sodium hydroxide (NaOH) at 110°C for three (3) hours at 300 RPM using a magnetic stirrer (Corning PC-420D). The deproteinized materials was then filtered and washed with distilled water until it reached a neutral pH. After DP, unbleached chitin was obtained. Unbleached chitin was treated with a solution of 5% sodium hypochlorite for 30 minutes at room temperature. The bleached samples were then filtered using a Whatman filter paper, washed to neutral pH with distilled water, and finally dried at 60°C for 24 hours. After this treatment, bleached chitin was obtained (Fig. 1). [2]
Assessment of the Chitin Purification Process
Samples were analyzed after the chitin purification process to determine changes in their chitin content, following the previous study.[23] The yield of bleached chitin was calculated as a ratio between the chitin dry weight and that of the initial insect (pupal exuviae) sample following the equation 1:

$$Chitin\ yield\ (\%) = \frac{dry\ weight\ of\ chitin\ (g)}{dry\ weight\ of\ the\ original\ raw\ \ pupal\ exuviae\ sample\ (g)} x100$$

Preparation of Chitosan
Chitosan was obtained from the bleached chitin extracted from the *H. celerio* L. pupal exuviae. Chitin samples were suspended in 50% sodium hydroxide (NaOH) under stirring for 4 hours at 80°C using a magnetic stirrer (Corning PC-420D) (300 RPM). At the end of the reaction, the suspension was filtered using filter paper, and the solid residue was washed to neutrality with distilled water. After washing, the deacetylated materials was incubated at 40°C for 24 hours. The yield of both chitosan, unbleached and bleached, were calculated for all the samples, similarly to chitin, according to the following equation 2: [23]

$$Chitosan\ yield\ (\%) = \frac{dry\ weight\ of\ chitosan\ (g)}{dry\ weight\ of\ the\ original\ raw\ pual\ exuviae\ sample\ (g)} \text{x}100$$

Chitin and Chitosan Characterization
All chitin and chitosan samples were analyzed using fourier-transform infrared spectroscopy (FTIR) and scanning electron microscopy (SEM) in order to characterize them and assess their quality and suitability for potential applications. Commercial chitin and chitosan isolated from shrimp shells were purchased and used to compare with the *H. celerio* L. pupal exuviae.
Fourier-transformed infrared spectroscopy (FTIR)
The IR transmission spectra (IRAffinity - 1S (Shimadzu) of the chitin and chitosan samples as well as the chitin and chitosan reference sample from crustacean shell were sent to the Natural Products Research and Development Center (Central Mindanao University) following the requirements for the preparation of samples for the analysis. Chitin and chitosan samples were characterized from 4,000 to 400 cm^{-1}. The degree of acetylation (DA) of chitin samples was determined by comparing the absorbance of the measured peak to that of the reference peak. The DA was calculated from the absorbance (A) ratios according to the following equation 3:[7]

$$Degree\ of\ Acetylation\ (DA)\ (\%) = \frac{A_{1655}}{A_{3450}}\ x\ 100$$

While the degree of deacetylation (DD) of chitosan was calculated according to the following equation 4:[24]

$$Degree\ of\ Deacetylation\ (DD)(\%) = \frac{\left[\frac{A_{1655}}{A_{3450}}\right]}{\frac{100}{1.33}}$$

Scanning Electron Microscopy (SEM)
Isolated chitin and chitosan, as well as reference samples, were sent to Ateneo de Davao University for analysis. The surface morphologies of the chitin and chitosan samples were examined by analyzing the powdered samples using scanning electron microscopy (SEM) (HITACHI SU-1510). The sample was coated with a gold/palladium alloy for 1 minute at 10 mA, and a sample-target distance of 15 mm.

Acute Oral toxicity
Test Animal
Twenty-one female Swiss albino mice, aged 8-12 weeks, and weighing 25-28 g were used in the study. They were maintained in the animal assay laboratory of Tuklas Lunas Development Center at Central Mindanao University at 24±1 °C, with a relative humidity of 55±5% under a 12-12 h light-dark cycle with food and water *ad libitum.*[25,26] They were housed in a polypropylene cage, with corn cob as bedding material. All mice (n=21) were acclimatized for one (1) week prior to the conduct of the acute oral toxicity. Prior to gavage, mice were on empty stomachs for the night before the activity while allowing water throughout the experiment.[27] The acute toxicity study was carried out in compliance with the Organization for Economic Cooperation and Development's (OECD) Test guideline No. 423 Acute Oral Toxicity, which governs chemical testing.[46] The mice were randomly assigned into seven groups, with each group consisting of three replicates. The initial body weights of the mice were noted. A single oral dose of 300mg/kg bw was given first. After 12 hours of observation and no mortality occurred, two additional concentrations 2000 and 5000 mg/kg bw of chitosan, were administered by gavage. Within four hours of treatment, for up to twenty-four hours, and then every day for fourteen days following treatment, the pertinent clinical signs were closely observed for any aberrant changes or mortality. Changes in body weight and behavior were recorded daily.

Statistical Analysis
Every measurement was done three times, and the average ± standard deviation was used to represent the results. A completely randomized design (CRD) was employed in the study, with replicates for each treatment group. Statistical Package for the Social Sciences (SPSS) (IBM Corp., 2017, version 25.0) was used to determine the differences among the values of various experimental groups. Data were expressed as means +- SD, and a P value <0.05 was considered statistically significant.

Proper Disposal of Carcass
The euthanized test animals were placed inside a plastic bag with proper labeling, such as the Institutional Animal Care and Use Committee's (IACUC) number, euthanasia method, date of experiment, and name of the researcher in accordance with the IACUC rules and protocols. The plastic bag was disposed in a designated bin intended for carcasses. Subsequently, the bag containing the animals was buried in a pit 3 feet below the ground.

Results and Discussion

Chitin and Chitosan Content
This study investigated the extraction of chitin and chitosan from *H. celerio* L. pupal exuviae, initially weighing 10 grams when dry, using three replicates to ensure the reliability of the methods. As shown in Table 1, the mean chitin yield was determined to be 21.74±2.15%, with an average extraction of 2.17 grams per 10 g sample. This finding indicates that chitin constitutes approximately one-fifth of the original dry weight of the pupal exuviae, demonstrating its substantial presence in these biological structures. The observed standard deviation of 3.73% suggests inherent variability in chitin yields, possibly influenced by the natural variability in pupal exuviae composition or nuances in the extraction process. Similarly, the mean chitosan yield was found to be 58.26±4.24%, with an average extraction of 1.28 grams per 10 g sample. This equates to chitosan comprising about three-fifths of the original dry weight of the *H. celerio* L. pupal exuviae. The standard deviation of 7.34% among chitosan yields indicates variability across samples. The standard error of the mean (SEM) values (0.21 for chitin and 0.19 for chitosan) underscores the precision of these estimates, ensuring confidence in the reliability of the mean values obtained from the replicates.
The present findings on the yield of chitin obtained from the pupal exuviae of *H. celerio* L. slightly differ from the findings reported by a previous study using insects a a source. Chitin content for *Calliptamus barbarus* belonging to order Orthoptera was 20.5±0.7%,[28] *Agabus bipustulatus* (Coleoptera) (14-15%),[29] *Tenebrio molitor* (Coleoptera) (13.3%- 17.7%),[7] *Musa domestica* (Diptera) from pupa shells with 8.02%,[30] *Hermetia illucens* (Diptera) bleached pupal exuviae with 23±1.9%,[23] *Caribena versicolora* (Araneae) exoskeleton with 19%,[31] *Achatina* shells (74.64%),[32] *Bombyx mori* (Lepidoptera) larva cuticle and silkworm pupa exuviae were reported to yield 15-20% of chitin.[33] The chitin content of the dry weight for *H. celerio* L. pupal exuviae was found to be close to that of the commercially utilized crabs and shrimp, which was found to vary from 17% to 20% of the dry weight.[34]
The results of this investigation are in agreement with those published by multiple authors,[29,30,23] who showed that a decrease in chitin yield could result from an extraction sequence that begins with demineralization (DM), deproteination (DP), and depigmentation. This occurs as a result of DP eroding the protein layer covering the chitin matrix before DM, which exposes it to acidic treatment and significantly removes inorganic material. Low chitin output was the result of significant chitin fraction loss and hydrolysis.[35] The chitin content of different insect species, genera, families, orders, and classes might also vary.[36] Furthermore, it was noted that the development phases and the insect's holometabolous or hemimetabolous status affect the chitin output.[23] Since the chitin composition of insects and crustaceans is similar, it can be assumed that insects constitute a possible source of chitin.
The chitosan yield extracted from *H. celerio* L. pupal exuviae is 58.26±4.24% lower than previously reported studies. In class Insecta, the highest content of chitosan extracted was registered for the order Lepidoptera (73-97%), followed by Orthoptera (74-82%), Coleoptera (67-74%), Hemiptera (69-70%) and Odonata (67 %).[36] The chitosan yield for *Achatina* shells was 58.60%, close to the yield of *H. celerio* L. chitosan.[32] The lower yield of *H. celerio* L. chitosan might be attributed to the chitosan depolymerization or to excessive removal of acetyl groups from the polymer during deacetylation, which results in sample weight loss, or from the loss of chitosan particles during sample washing to ensure sample neutrality.[38]
These results underscore the potential of *H. celerio* L. pupal exuviae as a sustainable source of chitin and chitosan for various industrial applications, highlighting the importance of refining extraction protocols to optimize yield and quality in biotechnological and material sciences. The degree of purity of bleached chitin extracted from different insect species mostly ranges from 85 to 97%. Given the same insect biomass, the observed differences in chitin purity may be due to variations in purification methods, which include reagents used, concentrations, and reaction times.

Chitin and Chitosan Characterization
The FTIR technique was used to perform physicochemical characterization of chitosan to determine the degree of acetylation and deacetylation process and to identify the functional groups present in the material.

Fourier-transformed infrared spectroscopy (FTIR) of isolated chitin
Spectra resulting from FTIR analysis of both unbleached and bleached pupal exuviae of *H. celerio* L. and commercially available chitosan and chitin from shrimp are shown in Fig. 2. All the characteristic peaks of bleached and unbleached chitin were detected in all samples at their specific wavelengths: 3593.38 cm^{-1} & 3485.37 cm^{-1} (OH-stretching), 3257.77 cm^{-1} (NH asymmetric stretching), 3101.54 cm^{-1} & 3103.46 cm^{-1} (NH-symmetric stretching), 1558.48 cm^{-1} (NH-bending, amide II), 1317.38 cm^{-1} & 1315.45 cm^{-1} (CN-stretching, amide III). The -α- form was confirmed for all chitin samples produced from *H. celerio* L. pupal exuviae by observing the two Amide I (C=O, stretching) band splits at 1622.13 cm^{-1} and 1658.78 cm^{-1} (bleached chitin), 1622.13 cm^{-1} and 1658.78 cm^{1} (unbleached chitin). Certain variations in peak wavelengths are most likely caused by variations in natural sources and the extraction technique.
Table 2 explains the assignments of the relevant bands from IR spectra of commercial chitin from shrimp (standard/reference) and *H. celerio* L. pupal exuviae. The spectra of all chitins showed structural similarity with the commercial polymer. These findings were similar to the previous study where author used chitin from black soldier flies and obtained the same characteristic peaks.[35] These results are also consistent with the findings of Kim *et al.*[30] in which the authors used chitin from *Musa domestica* pupa shells and obtained the same characteristic bands. Furthermore, a band was observed at 896 cm^{-1}, which was noted in earlier investigations.[35] This peak indicates the existence of the glycosidic bond, an alpha-chitin characteristic band that was found in all chitin samples (Fig. 2). The absence of a band at 1540 cm^{-1} demonstrated no protein residues in the chitins, indicating the successful deproteination process.[39]
If the degree of acetylation (DA) value of chitin is greater than 100%, some mineral residues could be present in the chitin. If the DA value of chitin is much less than 100%, protein residues in the chitin may be present. In this study, the DA value for bleached chitin is 104%, which is close to 100%, showing that the extracted chitin was close to pure. On the other hand, the unbleached chitin, showed 144%, indicating the presence of mineral residues. In earlier studies, the DA values for chitin isolated from different organisms were determined to be 102% for cicada sloughs, 104% for rice-field crab shells, 87% for bumblebees, and 151% for crude chitin from crabs.[28] Commercial chitin from shrimp has a DA of 123%. Order Orthoptera has the highest DA value, which ranges from 109%-232%, while the lowest DA value belongs to order Blattodea.[36] None of these DA values reported in earlier papers were

100%, indicating that some mineral or protein residues were still present in those chitin samples.

Fourier-transformed infrared spectroscopy (FTIR) of isolated chitosan
Spectra resulting from FTIR analysis of chitosan samples are shown in Fig. 3 in comparison with the commercial one. As reported for chitin, characteristic peaks confirming the identity of chitosan were detected, specifically the NH-bending (amide II) and CO-stretching (amide I) bands around 1649 cm^{-1} (amide I) and 1587 cm^{-1} (NH_2 bending), respectively. The characteristic bands were recorded at 1649 cm^{-1} and 1587 cm^{-1} for the chitosan from *H. celerio* L. pupal exuviae (bleached and unbleached) and for commercial chitosan. The characteristic peaks observed at 1649 cm^{-1} in both *H. celerio* L. and standard commercial chitosan from shrimp were due to the presence of amide I (C=O) in the acetamide group ($NHCOCH_3$), while the peaks observed at 1587 cm^{-1} in both chitosan samples were due to the amide II band (NH_2) in the $NHCOCH_3$ group. These present findings are consistent with those reported in the previous study, where the author reported similar characteristic peaks using chitosan from BSF larvae and adult flies,[35] and for silkworm chrysalis and orthoptera (*Calliptamus barbarus*).[28] The previous investigations showed that the peaks at around 1650–1655 cm^{-1} and 1583–1590 cm^{-1}, which correspond to (C=O) in the $NHCOCH_3$ group (amide I band) and (NH_2) in the $NHCOCH_3$ group (amide II band), respectively, were characteristic of chitosan.[23]
The degree of deacetylation (DDA) in *H. celerio* L. bleached is 86%, which is slightly higher compared to the DDA of commercial chitosan (83%). Several studies of insects showed comparable results with our study. DDA of Orthoptera ranged from 57-91%, Lepidoptera (81%), and Diptera (89-90%).[36] DDA is an important parameter for determining the quality of chitosan. The higher the purity of the chitosan, the higher the DDA. This parameter also directly affects the biological, functional, and physicochemical properties of the obtained chitosan. Furthermore, the DDA is used to indicate the effectiveness of the chemical deacetylation process for removing acetyl groups.[38] To clarify the underlying processes at play in this animal model, acute oral toxicity was further assessed in the current work.

Table 1: Summary of chitin and chitosan yields from *H. celerio* L. pupal exuviae

	Chitin (g)	Chitin Yield (%)	Chitosan (g)	Chitosan Yield (%)
Mean	2.17	21.74	1.28	58.26
Standard deviation	0.37	3.73	0.33	7.34
Standard Error Mean(SEM)	0.21	2.15	0.19	4.24
Mean ±SEM	2.17 ± 0.21	21.74± 2.15	1.28 ± 0.19	58.26± 4.24

Table 2: Functional groups with their corresponding % transmission and wavenumber of chitin from *Hippotion celerio* L. pupal exuviae

Functional groups	*H. celerio* L. Pupal Exuviae (Bleached)		*H. celerio* L. Pupal Exuviae (Unbleached)		Commercial Chitin (Shrimp)	
	% Transmittance	Wavenumber (cm^{-1})	% Transmittance	Wavenumber (cm^{-1})	% Transmittance	Wavenumber (cm^{-1})
OH-stretching	70.02	3593.38	80.94	3485.37	66.89	3479.58
NH-asymmetric	64.79	3257.77	78.40	3257.77	64.46	3259.70
NH-symmetric stretching	68.52	3101.54	81.66	3103.46	65.16	3101.54
C=O-stretching, amide I	62.74	1622.13	75.89	1622.13	63.13	1624.06
Split in two if alpha chitin	63.27	1658.78	73.91	1658.78	62.48	1647.21
NH-bending, amide II	61.34	1558.48	74.78	1558.48	60.38	1558.48
CN-stretching, amide III	66.08	1317.38	79.95	1315.45	62.79	1311.59
Glycosidic bond	71.75	896.90	86.36	896.90	68.96	896

Scanning Electron Microscope (SEM) of isolated chitin
The surface morphologies of chitin and chitosan produced from *H. celerio* L. pupal exuviae were observed by SEM, one of the useful methods for visually verifying the morphology and physical condition of the chitin and chitosan surface. The surface morphology of chitosan and chitin found in insects varies depending on the source organism.[1] Four distinct surface morphologies for chitin have been described by several studies: (1) a dense, rough surface devoid of pores and nanofibers, (2) a surface that combines pores and nanofibers (the most (common morphology), solely fibrillar (3) and porous (4) surfaces.[23]
First, the surface morphology of the pupal exuviae prior to chitin purification at 1.00 mm shows several pores. As magnification

increases, the structure becomes more rough and evident openings (Fig. 4). Bleached and unbleached chitin extracted from the pupal exuviae of *H. celerio* L. were compared to the commercially available chitin isolated from shrimp shells. Magnification at x50 (1.00 mm) revealed that *H. celerio* L. chitin is denser, rough, and flaky in structure compared to the shrimp chitin. Upon closer examination (x1000–x10,000), all chitin samples displayed slight notable surface variations (Fig. 5). Chitin from pupal exuviae (both bleached and unbleached) at x10,000 magnification has a smooth, porous surface structure without

any chitin fibrils which is with similar morphology to silkworm chitin, cricket flour, and wheat beetle. [17, 40,18] While shrimp chitin has a fibrous structure with pores which corresponds with the previous research. [1, 41] This features can be seen in alpha chitin, repeating polymer units that contain acetyl groups on opposite sides, and that alternate their position to each monomer. [42] Bleaching treatments had not significantly affected the chitin morphology of pupal exuviae. According to its morphology, chitin can be used for different applications; particularly, the porous structure can be used in tissue engineering, biomedicine, and drug delivery, highly porous structure increases the accessible surface area and thus the adsorption capacity of the material.[16],[23] Fibrillary surfaces are suitable for the textile industry.[23]

Scanning Electron Microscope (SEM) of isolated chitosan

The *H. celerio* L. pupal exuviae chitosan at x50 had a flake-like structure (Fig. 6). As magnification increases (x1000-x5000), the surface appeared to be smooth with fibrillar structure and non-porous. The changes on the surface demonstrates that the deacetylation step altered the chitin structure.[23] The smooth surface of chitosan has been observed for chitosan from other species, such as cicadas, grasshoppers and deep sea mud shrimp.[40,43] Fibrillar structure and non-porous chitosan were observed in six aquatic invertebrates.[44] Commercial shrimp chitin at x50 is denser, and as magnification increases, it has a smooth surface without fibers and or/ pores. This result coincides with the previous study were chitosan was extracted from shrimp.[35] *H. celerio* L. pupal exuviae chitosan surface appearance is different from black soldier fly pupal exuviae that had rough surfaces without the presence of any fibers and/or pores.[35] According to earlier research, the surface morphology of chitosan differs depending on the organism. [44] Their distinct intra-sheet/ intersheet or hydrogen-bonding systems may also be the cause of the variations in the crystallinity structure.[43] Surface morphology is one of the vital properties that determines the effective use/ application of chitin and chitosan.

Acute Oral Toxicity Assessment of Isolated Chitosan

The safety of insect-derived chitosan was examined using acute oral toxicity testing on Swiss albino mice. The animals received a starting dose of 300 mg/kg bw of chitosan produced from the pupal exuviae *H. celerio* L. and commercially available extracted from shrimp. During the four hours of observation, no detrimental effects on the animals were seen. In addition, each mouse received a higher dose of 2000 mg/kg bw, and they were monitored for 24 hours. Again, no adverse effects or animal deaths were documented. Furthermore, the acute toxicity test was carried out at a higher dose of 5000mg/kg bw in accordance with OECD 423 criteria. Oral administration of chitosan at various dosages in Swiss albino mice resulted in no clinical changes, toxic symptoms, or mortality during the study period, even the maximum tested dose of 5000 mg/kg bw. The median lethal dose (LD_{50}) of chitosan from *H. celerio* L. pupal exuviae is clearly defined and measured at more than 5000 mg/kg bw.

This study investigated the impact of varying doses of chitosan on the weight gain of Swiss albino mice over a 14-day period, comparing results to a control group (Fig. 7). Seven groups were included in this study: a control group and groups receiving CS 300 mg/kg, CS 2000 mg/kg, CS 5000 mg/kg, CPE 300 mg/kg, CPE 2000 mg/kg, and CPE 5000 mg/kg. Each group's weight measurements were recorded daily from Day 0-14 to analyze changes in mice weights in response to the administered substances. The control group given with vehicle only (0.9%NaCl) demonstrated consistent weight gain, starting at 25.93 ± 0.61 grams on Day 0 and progressing to 28.1 ± 0.35 grams by Day 14, resulting in a measured weight gain of 2.17 ± 0.65 grams. In contrast, the groups treated with the chitosan at varying dosages exhibited distinct responses. The group administered 300 mg/kg of chitosan shrimp (CS) showed a significant decline in weight, starting at 25.83 ± 0.12 grams on Day 0 and decreasing steadily to 23.1 ± 0.68 grams by Day 14, indicating a notable weight loss of -2.73 ± 0.34 grams (*p < 0.05 compared to control). Similarly, the 2000 mg/kg (CS) group also experienced a reduction in weight, starting at 25.57 ± 0.32 grams and decreasing to 24.6 ± 0.25 grams by Day 14, with a weight loss of -1.0 ± 0.15 grams (*p < 0.05 compared to control). Additionally, the 5000 mg/kg (CS) group also decreased in weight, starting at 25.57 ± 0.32 grams and decreasing to 24.6 ± 0.25 grams by Day 14, with a weight loss of -1 ± 0.15 grams (*p < 0.05 compared to control). The groups treated with CPE (300 mg/kg, 2000 mg/kg, and 5000 mg/kg) all exhibited significant decreases in weight gain compared to the control group, with reductions ranging from -2.57 ± 0.28 grams to -3.13 ± 0.37 grams (*p < 0.05 for all treated groups compared to control) (Table 3). These findings emphasize the significant effects of the commercial chitosan and chitosan isolated from the pupal exuviae of *H. celerio* L. species, particularly evident at higher doses and across different formulations, in reducing the weight gain of mice over the 14-day experimental period. The chitosan in a mice study could inhibit fat digestion, dissolve in acidic gastric juices, and act as an emulsifier on fat globules which results in increased fat excretion in feces, approximately 7.5 times higher compared to that of a cellulose-fed group.[45] Our results are consistent with other studies that found chitosan reduced body weight fed on mice. [48] This suggests that chitosan is a good supplement for diet.

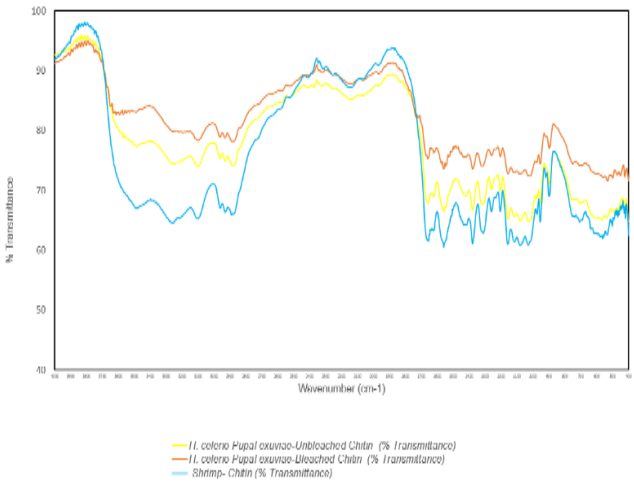

Figure 2: FTIR spectra of chitin extracted from the pupal exuviae of *H. celerio* L. compared to commercially available chitosan from shrimp shell

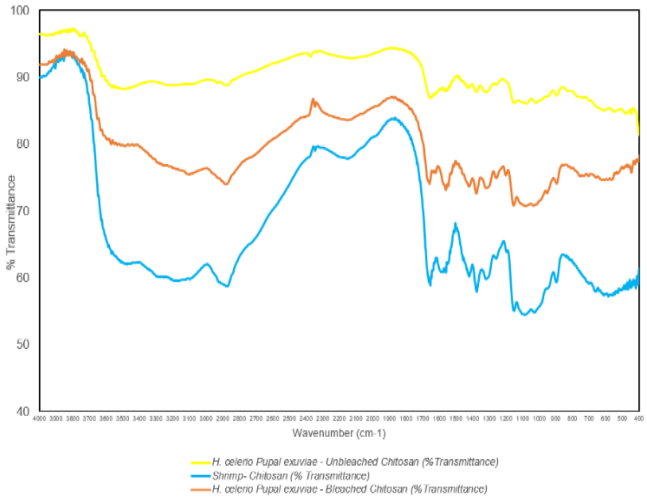

Figure 3: FTIR spectra of chitosan extracted from the pupal exuviae of *H. celerio* L. compared to commercially available chitosan from shrimp shell.

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Table 3: Mean body weight of mice (g) observed during 14 days of observation of feeding chitosan in their diet

Treatment	Day 0 (Initial weight)	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14 (Final weight)	Weight gain
Control	25.93 ± 0.61	26.07 ± 0.55	26.1 ± 0.49	26.3 ± 0.53	26.5 ± 0.7	26.6 ± 0.78	26.9 ± 0.81	27.1 ± 0.92	27.3 ± 27.3	27.4 ± 0.75	27.6 ± 0.7	27.7 ± 0.72	28 ± 0.47	28.1 ± 0.35	28.1 ± 0.35	2.17 ± 0.65
CS (300 mg/kg)	25.83 ± 0.12	25.4 ± 0.3	24.9 ± 0.4	24.7 ± 0.3	24.4 ± 0.46	24.2 ± 0.3	24 ± 0.3	23.9 ± 0.35	23.8 ± 0.4	23.8 ± 0.21	23.5 ± 0.21	23.5 ± 0.25	23.5 ± 0.3	23.4 ± 0.4	23.1 ± 0.68	-2.73 ± 0.34[a]
CS 2000 mg/kg	26.57 ± 0.31	26.03 ± 0.06	25.9 ± 0.15	25.7 ± 0.26	25.5 ± 0.26	25.3 ± 0.15	25.2 ± 0.17	25.1 ± 0.21	25 ± 0.15	24.9 ± 0.15	24.8 ± 0.29	24.9 ± 0.26	24.9 ± 0.29	24.9 ± 0.55	24.9 ± 0. 61	-1.67± 0.27[a]
CS 5000 mg/kg	25.57 ± 0.32	25.13 ± 0.40	24.5± 0.74	24.2 ± 0.79	24.3 ± 0.68	24.2 ± 0.57	23.9 ± 0.21	23.9 ± 0.25	23.7 ± 0.3	23.9 ± 0.35	24.2 ± 0.51	24.2 ± 0.57	24.3 ± 0.4	24.4 ± 0.21	24.6 ± 0.25	-1± 0.15[a]
CPE 300 mg/kg	26.63± 0.21	25.97 ± 0.46	25.8 ± 0.55	25.5 ± 0.45	25.5 ± 0.36	25.3 ± 0.26	25.1 ± 0.36	25 ± 0.46	24.6± 0.52	24.2 ± 0.51	24.1 ± 0.46	23.8 ± 0.55	23.7 ± 0.61	23.6 ± 0.55	23.5 ± 0.52	-3.13 ± 0.37[a]
CPE 2000 mg/kg	26.13 ± 0.15	25.63± 0.11	25.3 ± 0.15	25 ± 0.3	24.8 ± 0.2	24.6 ± 0.21	24.4 ± 0.67	24.1 ± 0.87	24 ± 0.87	23.9 ± 0.83	23.9 ± 0.71	23.8 ± 0.56	23.5 ± 0.57	23.5 ± 0.53	23.4 ± 0.31	-2.7± 0.30[a]
CPE 5000 mg/kg	25.93 ± 0.25	25.43 ± 0.45	25.3 ± 0.45	25 ± 0.25	24.9 ± 0.32	24.9 ± 0.25	24.7 ± 0.20	24.6 ± 0.30	24.4 ± 0.11	24.1 ± 0.17	24.1 ± 0.31	24 ± 0.29	23.7 ± 0.44	23.5 ± 0.56	23.4 ± 0.5	-2.57 ± 0.28[a]

Data provided as mean±SEM (n=3) in Day 0 to Day 14; [a]p <0.05 treated groups Versus control
*CPE- chitosan pupal exuviae
*CS- chitosan shrimp (Commercially available)

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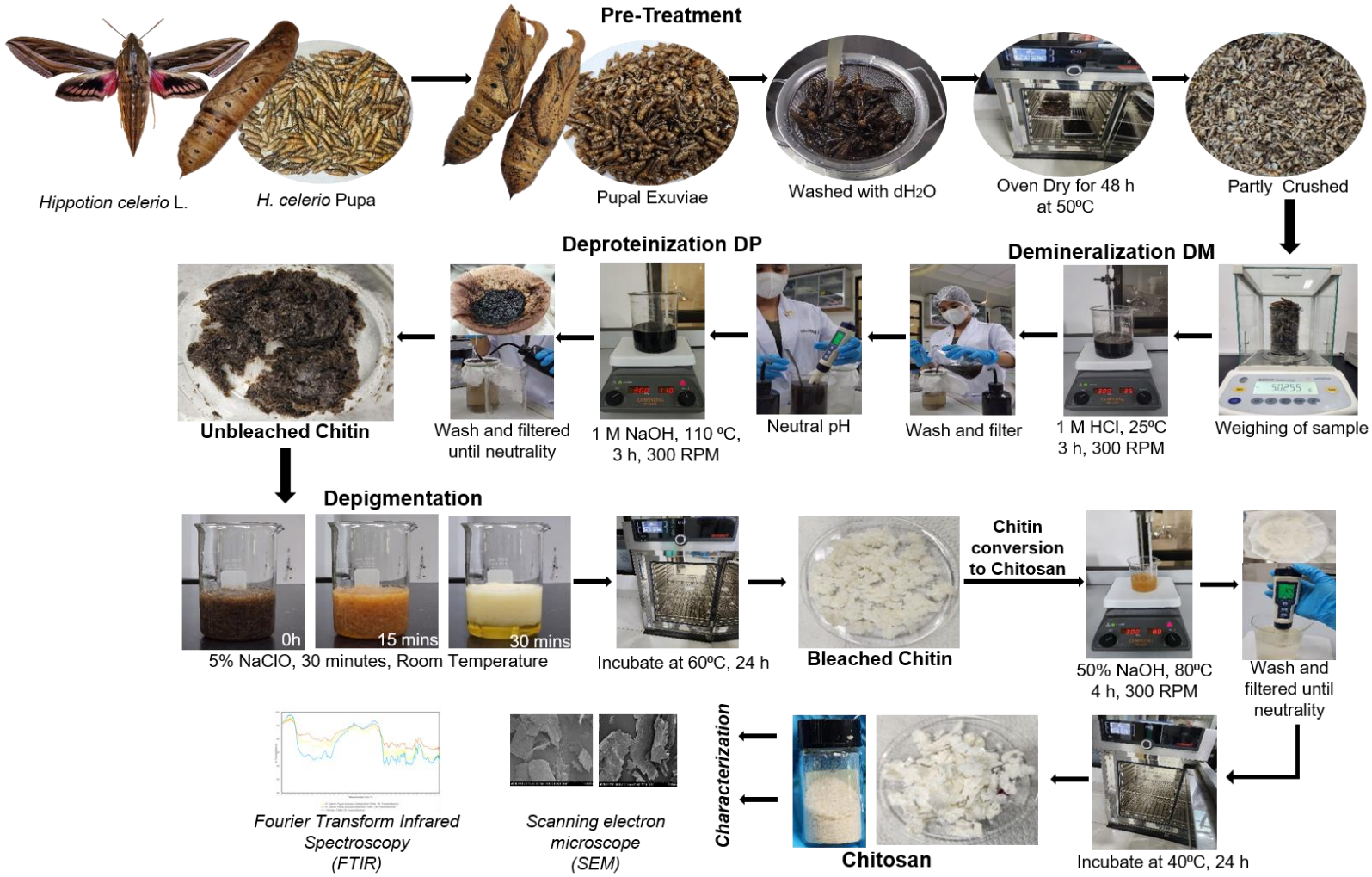

Figure 1: Schematic diagram of Chitin and Chitosan purification from the pupal exuviae of *H. celerio* L.

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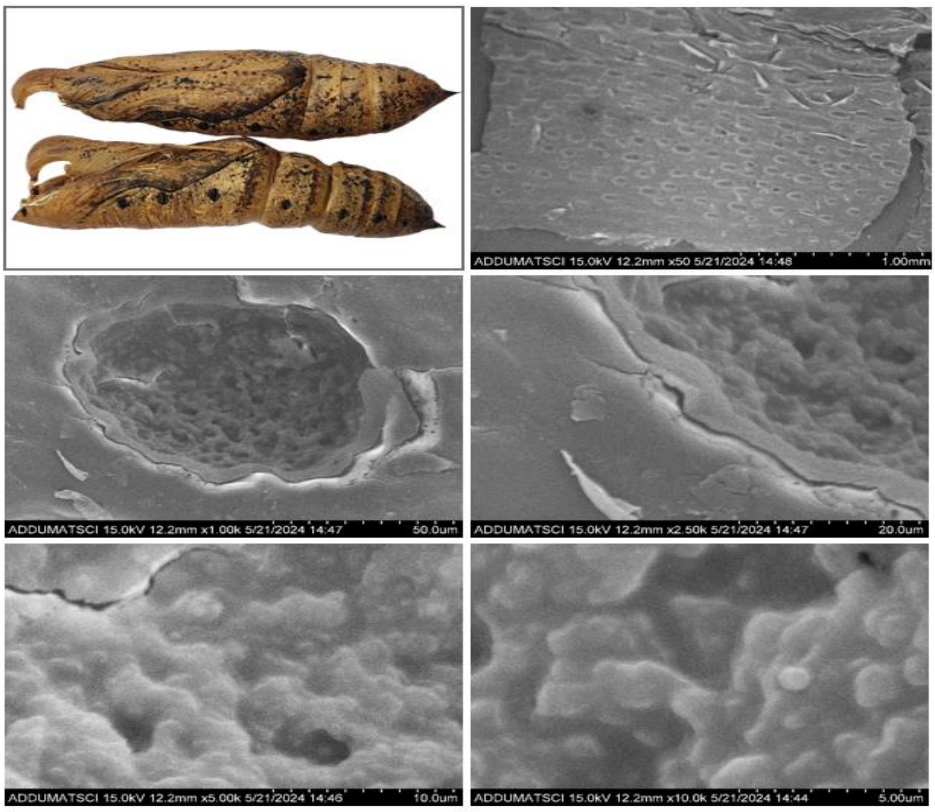

Figure 4: Scanning electron micrograph of the surface morphology of *Hippotion celerio* L. pupal exuviae

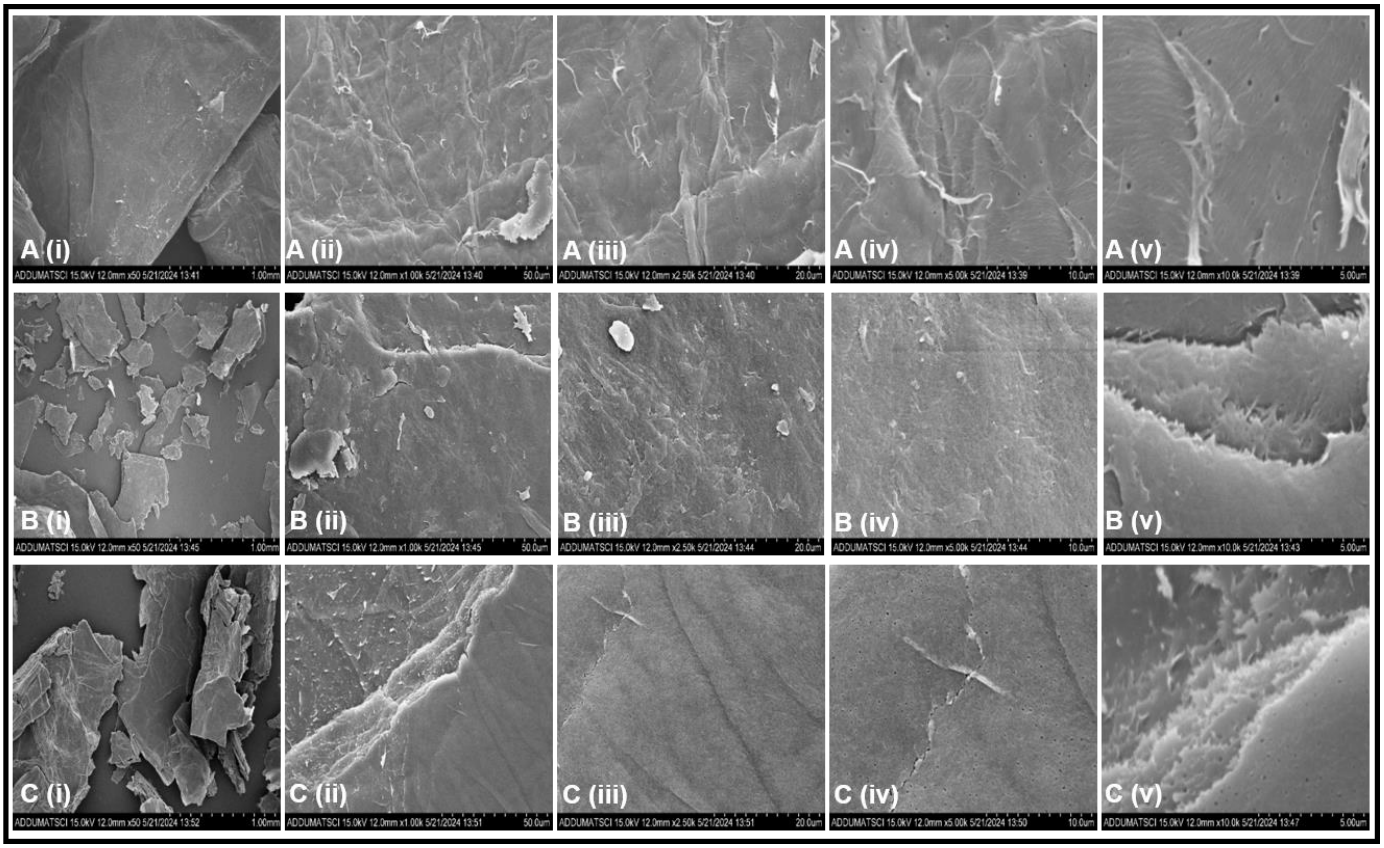

Figure 5: SEM images of **(A)** Chitin Shrimp **(B)** *H. celerio* L. Unbleached Chitin **(C)** *H. celerio* L. Bleached Chitin (i-x50 1.0mm; ii-x1000, 50 μm; iii- x2500, 20 μm; iv- x5000, 10 μm; v- x10000, 5 μm

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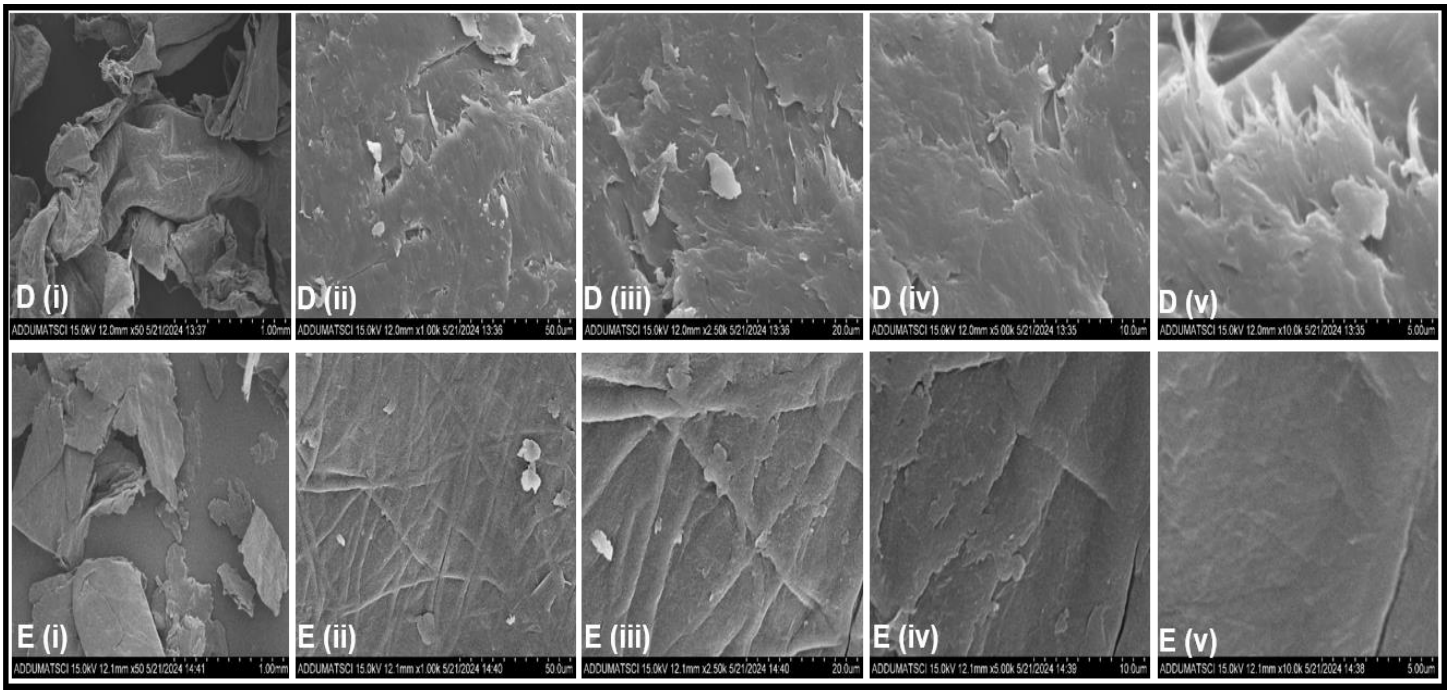

Figure 6: SEM images of D) Shrimp Chitosan E) *H. celerio* L. Chitosan (i-x50 1.0mm; ii- x1000, 50 µm; iii- x2500, 20 µm; iv- x5000, 10 µm; v- x10000, 5 µm.

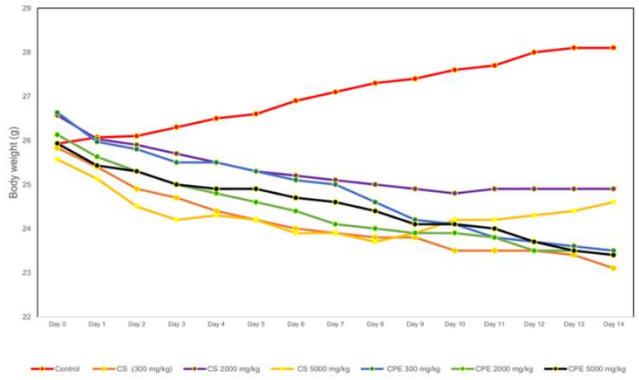

Figure 7: Mean body weight (g) of mice observed during 14 days of treatment of chitosan from *Hippotion celerio* L.

Conclusion

Therefore, the chitin and chitosan extracted from one of the pupal exuviae of hawkmoth species (*H. celerio* L.) are comparable with the commercially available chitosan extracted from shrimp in terms of % yield and degree of deacetylation. Furthermore, the surface morphology of chitin and chitosan, as analyzed by scanning electron microscopy, indicates potential uses for this polymer in biotechnology because of its fibrillar and porous structure. Overall, the chitosan obtained from pupal exuviae of *H. celerio* L. has no toxicity and may be relatively safe in mice as they did not cause any mortality or changes in general behavior for single-dose administration. Subsequent research endeavors will encompass the histological analysis and additional biomedical uses of insect-derived chitosan.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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