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Sustainable eco-friendly phytoextract mediated one pot green recovery of chitosan

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ABSTRACT-
Chitin and chitosan are biopolymers that have diverse applications in medicine, agriculture, food, cosmetics, pharmaceuticals, wastewater treatment and textiles. With bio-origins, they easily blend with biological systems and show exemplified compatibility which is mandatory when it comes to biomedical research. Chitin and chitosan are eco-friendly, however the processes that are used to recover them aren't eco-friendly. The focus of this work is to attempt an eco-friendly, sustainable phytomediated one pot recovery of chitosan from commercial chitin and from crab and shrimp shells and squid pen solid wastes. Graviola extracts have been employed, given the fact file that their active ingredients acetogenins actively interact with chitin in insects (resulting in its application as an insecticide). With that as the core idea, the graviola extracts were chosen for orchestrating chitin recovery and a possible chitin to chitosan transformation. The results confirm that graviola extracts did succeed in recovery of chitosan nanofibers from commercial chitin flakes and recovery of chitosan particles directly from solid marine wastes of crab, shrimp and squids. This is a first time report of a phyto-extract mediated novel chitosan synthesis method.

I. INTRODUCTION-

Chitin has also been discovered in fossils. Chitin is the benevolence of nature, as part of the exoskeleton of mollusks1, Arthropods2, 3, in the cell wall of mushrooms4, and corals5 and sponges6–9, and algae10, 11 body structures. Being the second most abundant polysaccharide on the planet after cellulose, chitin is just as much a valuable contribution from the animal kingdom as it is from the plant kingdom. Chemically, chitin is a linear polymer consisting primarily of 2-acetamide-2 -deoxy-β-D-glucopyranose unit's β-(1/4)-linked 2-amino-2-deoxy-β-D-glucopyranose units and partially of β-(1/4).

In nature, chitin exists in two allomorphic forms, α and β-forms and in addition as π-chitin (a mixture of structures α and β) 16. The alpha form17 is reportedly the predominant of these three. The concentration of chitin in shells of crabs and shrimps is 20% (percent of dry weight) and 31% in squid cages, the highest values in commonly available marine products. Other less common sources include cicada sloughs (36%), silkworm chrysalides (20%) and cyst shells (29%–34%) 6. Shells and krills of crab and shrimp remain the sources of chitin recovery that are used most. A chitin derivative, chitosan is a linear (1–4)-linked 2-amino-2-deoxy-β-D-glucopyranose monomer polysaccharide. It is made from the extracted chitin's deacetylation. The deacetylation of chitin using a solid alkaline medium has been considered as the main method of supplying chitosan20, 21. Chitosan is soluble in acidic solutions22, 23 when the degree of deacetylation (DDA) reaches higher than approximately 90%.

Besides these, there are various useful biological properties of chitin and its derivatives, primarily chitosan: biocompatible, biodegradable, antimicrobial, wound healing, and haemostatic25–27.

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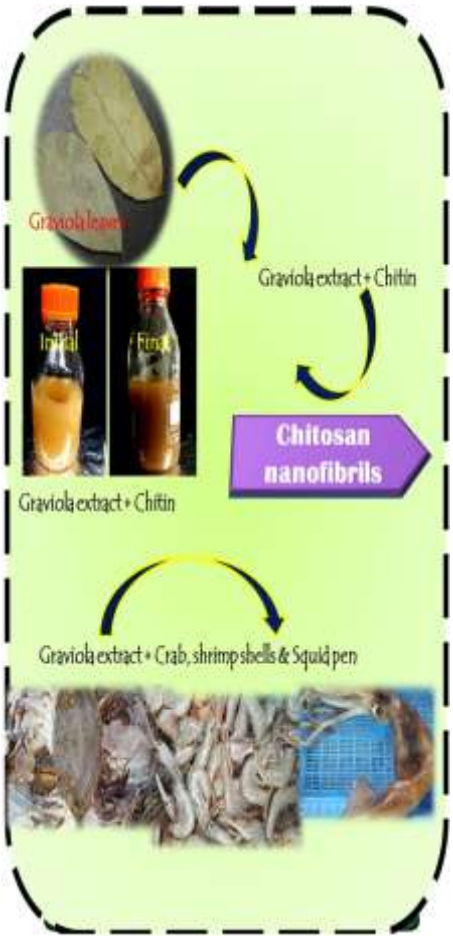

Figure 1. Schematic work flow of the study.

Chitin extraction typically flows through two stages; demineralization using HCl to eliminate calcium carbonate and deproteinisation using a protein removal NaOH solution. A number of major steps are involved in the most common method of chemical separation of chitin from crustacean shells: washing, grinding and sieving raw shells, followed by demineralization (elimination of calcium carbonate in dilute acid). Deproteinisation in aqueous NaOH or KOH follows this. Use of enzymatic hydrolysis for deproteinization and micro-organisms has been reported for both demineralization and deproteinizatio. The complicated procedure involved and the by-products produced during each stage of interaction are problems confronting processes of chitosan extraction. Also, the high susceptibility of chitosan to processing conditions (such as heating or freezing) can impose stress on its structure and cause polymer degradatio. Hence, newer methods and techniques have also been attempted, with the most efficient one being the microwave assisted extraction methodolog. Chitosan is a hot topic biomaterial that has excellent properties, but a global problem is that it is difficult to get a continuous and easily accessible supply.

Chitosan nanofibers from commercial chitin. Morphological characterization

Polyether's class acetogenin is present in plant family Annonaceae37–39. These consist of fatty acid chains of C32 or C34 with terminal ÿ-lactones, which also include groups of epoxide, hydroxyl, ketone, tetrahydrofuran and tetrahydropyrene.

Graviola is a member of the Annonaceae family, known for its anticancer function acetogenins. Acetogenins are also known for their activity of dechitinization, which has formed the

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basis for their use against pest larvae40.It is with this background, that the possibility for a positive interaction between chitin and graviola extract was conceived. Commercial crab chitin was subjected to extracts of graviola (GE), and samples were obtained after periods of 1 day, 2 day, 3 day, 5 day, 6 day, and 7 day. Figure 1 shows workflow schematics in this article. Using FESEM the morphology of the collected material has been imaged. The FESEM results as observed in Fig. 2, reveals a progressive trend in the extraction of nanofiber's from the source material, commercial crab chitin. The as-received chitin were micro sized flakes (Fig. 2a–c), following interaction with GE after 1 day (Fig. 2d), 3 day (Fig. 2e), 5 day

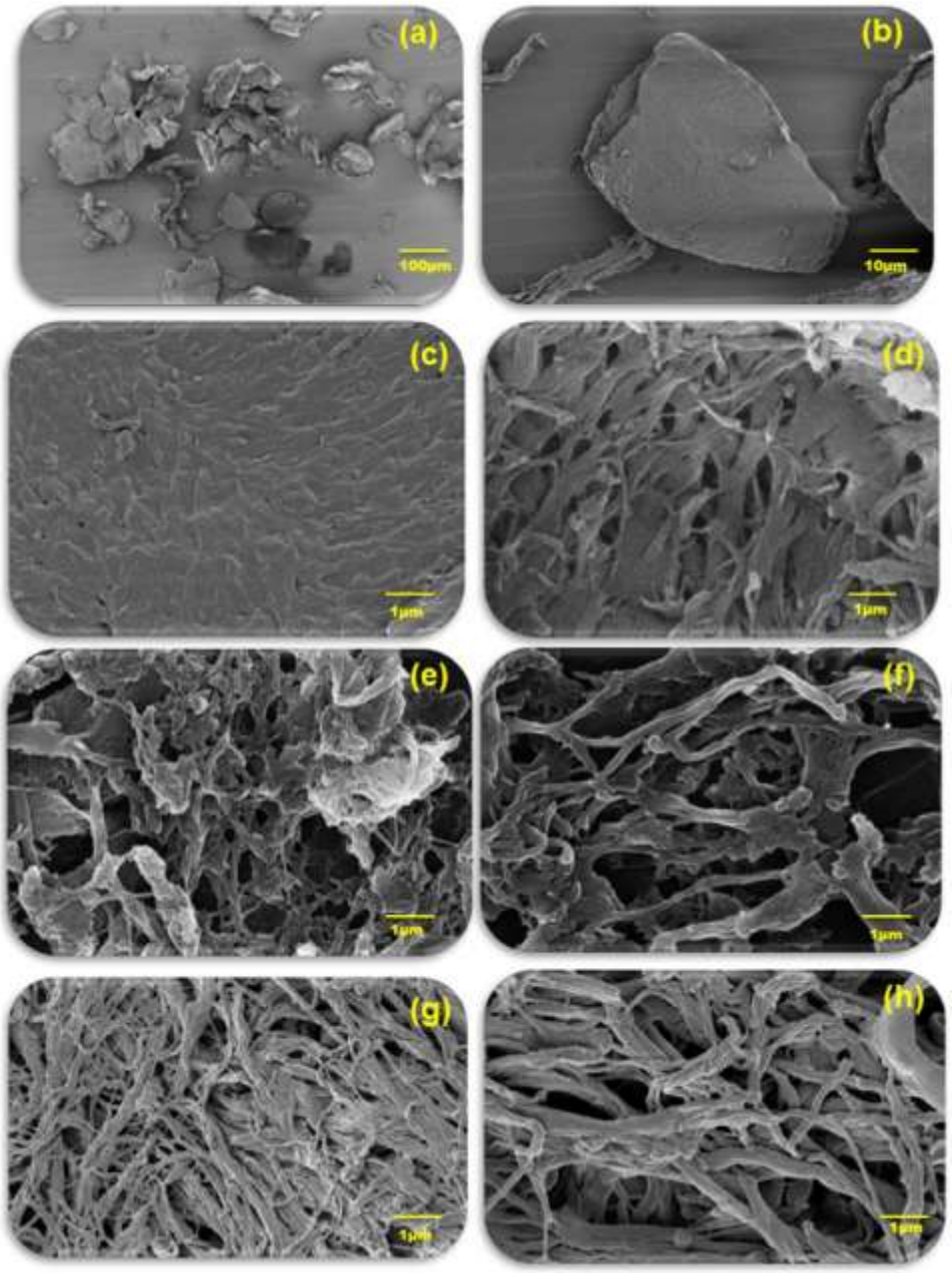

Figure 2. FESEM micrographs of (**a**,**b**) commercial chitin flakes incubated with GE for (**c**) 1 h (**d**) 1 day (**e**) 3days (**f**) 5 days (**g**) 6 days (**h**) 7 days.

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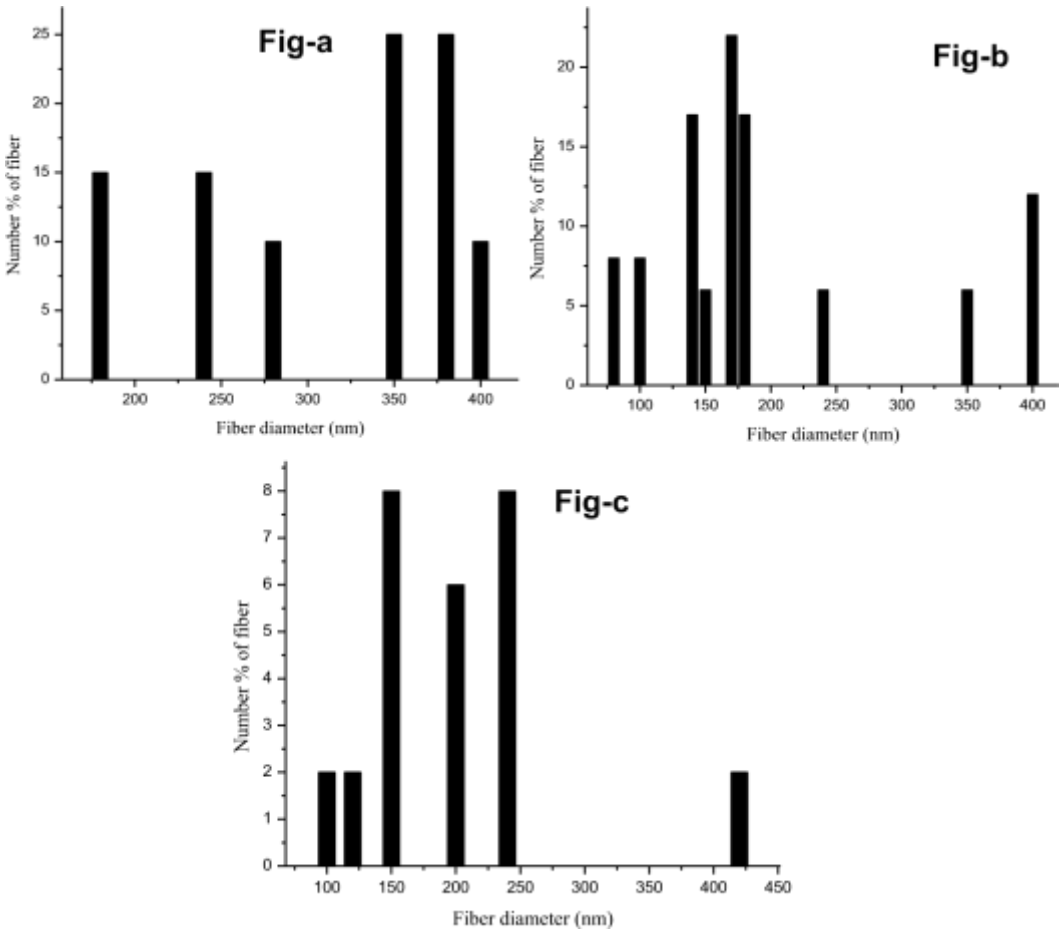
(Fig. 2f), 6 day (Fig. 2g) and 7 days (Fig. 2h) the nanofiber's started showing up. Nanofibers in the size ranges up to 100–200 nm were obtained. The fibre sizes were measured using OPTIMAS software based on the FESEM images. Figure 3 gives the size distribution histogram of the fibres obtained after 1 day (Fig. 3a), 3 day (Fig. 3b), 5 day (Fig. 3c), 6 day (Fig. 3d) and 7 days (Fig. 3e). As observed from the histograms, fibres obtained at 1 day were in the size range of 175–400 nm with sizes 350 nm to 375 nm predominating. As for fibres obtained at 3 day exposure conditions (Fig. 3a), the size ranges were 75–400 nm with sizes 170 nm to 180 nm predominating (Fig. 3b). 5 day (Fig. 3c) exposure conditions resulted in sizes of 150 nm predominating; in 6 day (Fig. 3d) exposures, size ranges of 75–200 nm (140 nm predominating) and in 7 day (Fig. 3e) conditions, 100–150 nm sizes were observed. As a result of heat treatment the dissolution of the protein matrix holding the chitin fibre's released the chitin fibrils. The reduction in the size of the fibres is due to the magnetic stirring effect41. The pH of the GE extract was 6.3 and remained unchanged when interacting with the chitin material.

Chemical characterization.

The chemical structure of nanofibers is not yet confirmed, as it is understood that chitin under specific conditions is deacetylated to form chitosan. The nanofibers that were obtained from chitin were thus characterized to guarantee their chemical identity. Figure S1 gives the nanofibers UV-Vis spectrum



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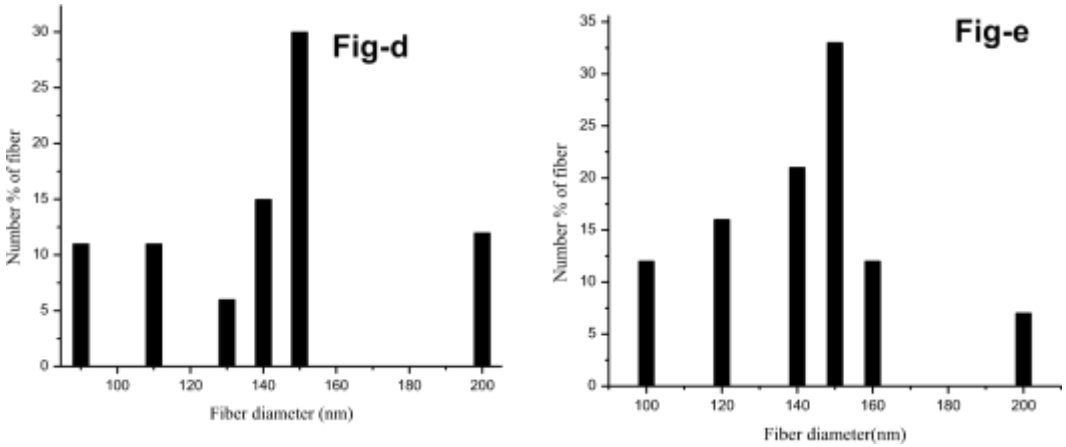

Figure 3. Size distribution histograms of chitosan nanofibers following incubation with GE for (**a**) 1 day (**b**) 3days (**c**) 5 days (**d**) 6 days (**e**) 7 days.

In water. As observed from the figure, standard chitin shows absorption in the 100 to 250 nm range same as that of chitosan. In this range spikes of varying intensity were observed in the nanofibers harvested from the GE extracts at different interaction time. The other peaks seen in the UV spectra are from components of the GE extract which show maximum absorption in the range of 200–230 nm. Based on the UV-Vis absorption peaks it is thus not definitive to conclude the nature of the nanofibers to be that of chitosan. But as observed from the spectra, it can be seen that the nanofibers also showed enhanced absorption in the 200–230 nm region compared to the standard chitin peaks, indicating functionalization of components from the extract on their surfaces. A sudden spike in the area of chitin-chitosan absorption was observed after an incubation period of 5 days, which shifted suddenly to a spike in the 220–230 nm zone.

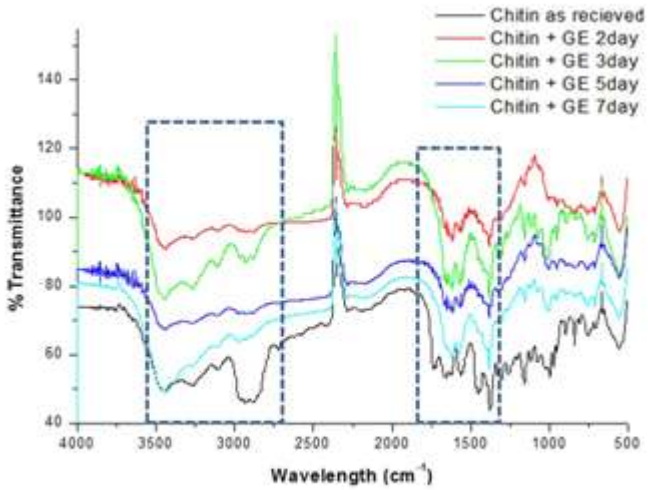

Figure4. FTIR spectra of control chitin and GE interacted chitin.

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Sample Name	Degree of deacetylation DDA %	Sample identity
Chitin + GE 1 day	50.97	Chitosan
Chitin + GE 3 day	94.86	Chitosan
Chitin + GE 5 day	92.73	Chitosan
Chitin + GE 7 day	86.37	Chitosan

Table 1. Degree of industrial Chitin deacetylation following contact with GE.

(210–225 nm) lying in the same area as that of chitin and chitosan, not much conclusions could be arrived at using the UV-Vis results alone.

FTIR is apparently a more conclusive evidence, since it clearly indicates chitin deacetylation with significant changes in the FTIR bands. Figure 4 shows the results of the FTIR investigations, the peak at 3417 cm^{-1} was stronger due to the hydroxyl group superimposed on the amine group stretching vibrations. The CH bond43 is representative of the highest at 2900 cm−1. Distinct changes in the region of 2900 cm−1 are apparent from Fig. 4. The GE interacted nanofibers show profound changes in this region as compared with standard chitin, indicating a transformation of chitin into chitosan. The 2-day at GE nanofibers show decrease in the 2900 cm−1 band that continued in the 3-day at GE nanofibers and increased up to 5-day GE nanofibers as a function of GE interaction. Moreover, the other peaks at 1659 cm^{-1} and the two peaks at 1382 and 1247 cm^{-1} were caused by CO stretching mode of carboxyl group conjugated to a NH deformation mode resulting in CONH, indicative of amide bond formation. The form of the bands between 1500 and 1750 cm−1 is also changed after deacetylation of the chitin major changes 1659 cm−1 undergoes changes and decay. Significant differences in the form of 2800–3500 cm−1 band (Fig. 4) can also be observed which confirms the transformation of chitin to chitosan44. Figure S2 shows the FTIR bands of standard chitosan. DDA which is the confirmative test for ensuring chitosan's identity was determined. As shown in Table 1, the DDA % obtained from the FTIR spectra of 2 day at GE nanofiber's, 3 day at GE nanofiber's, 5 day at GE and 7 Day at GE, confirmed the chemical identity of the nanofiber as chitosan. Consequently, the FTIR findings confirmed the chemical identity of the nanofibers obtained as being that of chitosan and not chitin. Moreover, these results indicated that prolonged incubation did not yield better results, rather a drop in DDA was observed at 7 day at GE, hence 3 day to 5 day incubation are considered optimal. Yield of chitosan was calculated by comparing the weight of the raw material to the weight of chitosan, which was obtained after the treatment. The average yield % at 3 day and 5 day periods was 55.34% ± 5.57. Different methods yielded varying results in literature, depending on the process, the extractant and the source material. Sarbon et al. recorded 44% of mud crab, Ibitoye et al., 4–10% of house cricket45, 46 and other authors report 72–75% from commercial chitin. A direct comparison could not be made between the traditional extraction methods and the new one, because chitosan extraction is subject to a great deal of variation.

Chitosan from marine solid wastes.

Morphological characterization.

In order to assess GE's ability to recover chitin / chitosan directly from shells, crab and shrimp solid shell wastes and squid pen were subject to direct one pot GE contact. The supernatant was screened for chitin / chitosan after 3 days of incubation with GE, and the shells were imaged using an inverted microscope. Fig. S3a–f summarizes these results. It has been found that the solid wastes turned black visually and in fact became extremely brittle. This brittle condition may result in chitin being the exoskeleton's structural structure, when inserted into the medium. Figure 4a shows the nature of the crab shell prior to interaction with GE and Fig. 4b shows the shell morphology following interaction. The insets show their respective photographs. In case of crab not much difference was observed, but in case

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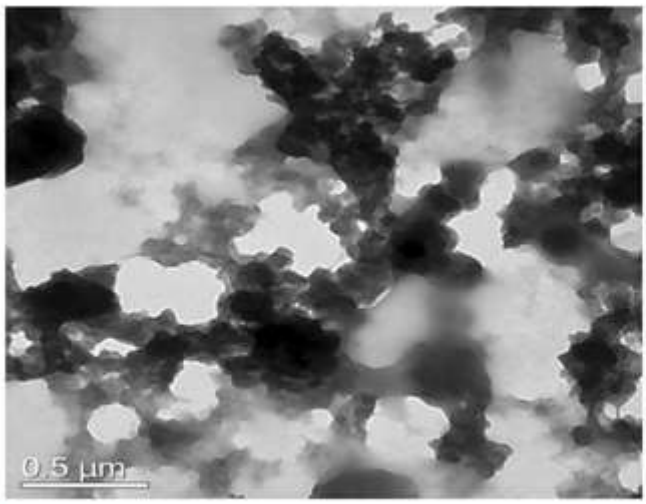

Figure5. TEM of chitos an recovered from shrimps hells following GE interaction of 3days.

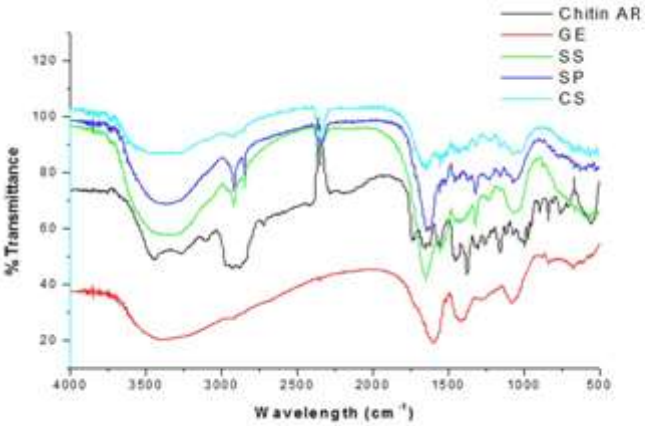

Figure6. FTIR spectra of chitos an recovered from crab and shrimps hells and squid pen.

Sample Name	Degree of deacetylation DDA %	Sample identity
Shrimp Shells (SS)	87.71	Chitosan
Squid Pens (SP)	82.11	Chitosan
Crab shell (CS)	72.73	Chitosan

Table 2. Degree of deacetylation of marine solid wastes following interaction with GE.

Of shrimp (Fig. S3d) and squid pens (Fig. S3f) distinct changes in the morphology was observed. In case of shrimp evident signs of removal of structural components was observed, resulting in the voids observed in the image. As for squid pens (Fig. S3f), clear alterations in the striations was observed in the control or as received pens (Fig. S3e) were evident. As the insets confirm, visible changes could be seen prior to (Fig. S3a, c,

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e) and following GE interaction (Fig. S3b, d, e) with respect to the external morphology,

The supernatant was characterized for chitosan particles, Fig. 5 endorses the morphology of the obtained material. As observed from Fig. 5, Particles obtained after contact with solid marine waste did not possess the morphology of nanofibers as observed in the above examples, but colloidal particulate morphologies.

Chemical characterization.

The UV Vis spectrophotometric results as shown in Fig. S4, Show suggestive chitin / chitosan absorption peaks in area 220–250 nm. In the case of squid pens the peaks were largest, followed by shells of crab and shrimp. All molecular chains in α-chitin are arranged in an antiparallel mode with strong intermolecular hydrogen bonding. B-chitin has a parallel chain packing with intermolecular forces weaker than those between chains of α-chitin. This feature makes β-chitin more vulnerable to chemical reaction or enzymatic degradation48. Many natural chitins have structure of α type, whereas chitin of the β type is less diffuse49. The calf pens are of β-type

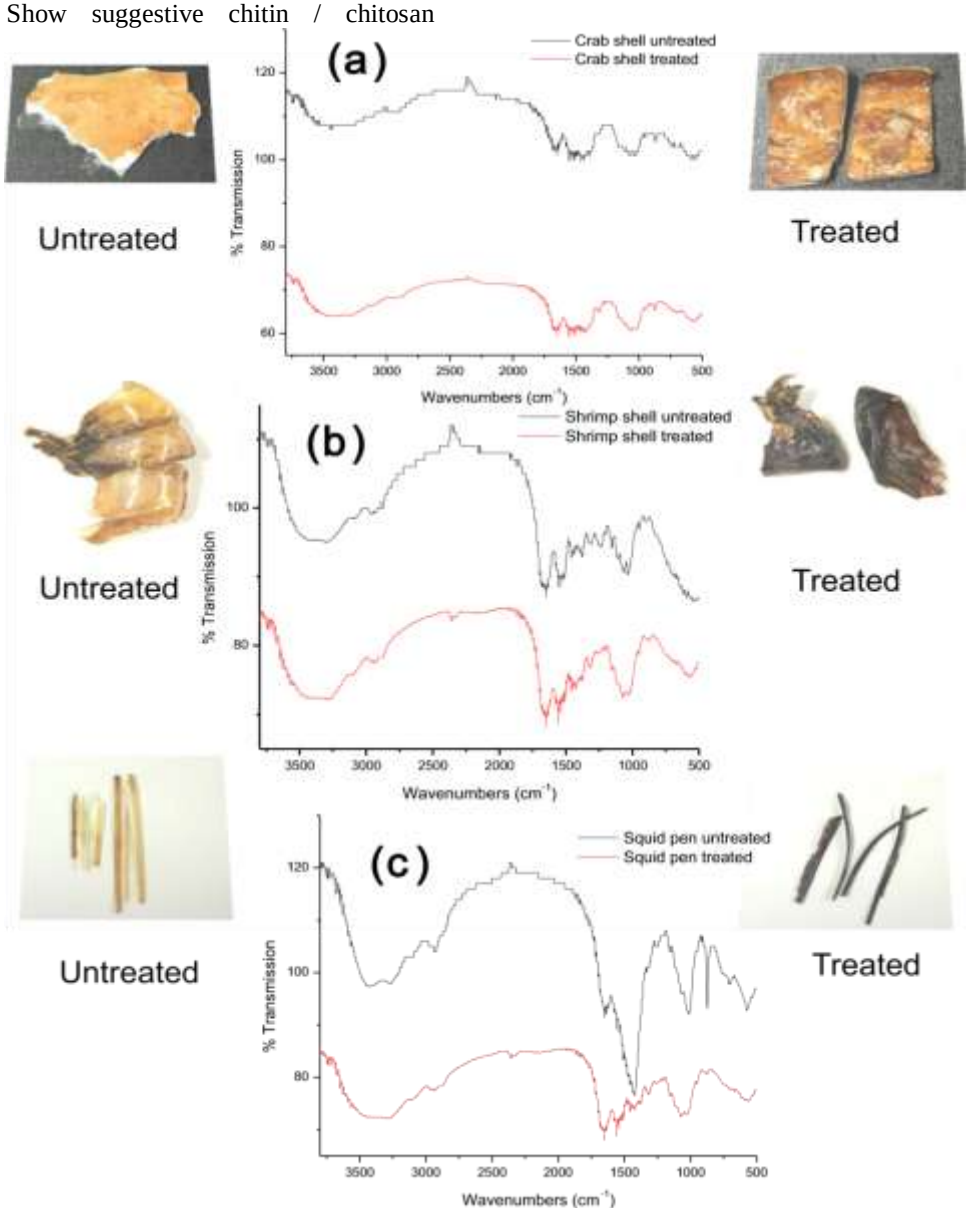

Figure 7. FTIR of (a) lobster, (b) shrimp, and (c) strong squid wastes before GE treatment and after exposure to GE.

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Chitin and, as it appears in our study, were those that enabled the easy and effective recovery of chitin / chitosan as shown by the high absorption of shrimp and crab shells. The yield percentage for SS, SP and CS respectively was 22.34 percent, 34.44 percent and 18.21 percent.

FTIR studies were undertaken and the results confirmed the recovery of chitosan from solid wastes. The recovered particles from the shells were confirmed to be that pertaining to chitosan and not chitin. Figure 6 clearly demonstrates characteristic chitosan bands on the FTIR spectra of the recovered material from crab shells, shrimp shells and squid pens. As shown in Table 2, the DDA % obtained from SS, SP and CS indicated the recovery of chitosan with 87%, 82% and 72% DDA respectively. So it is verified that the substance extracted from the solid marine wastes was not chitin but chitosan.

As supporting evidence that solid wastes on contact with Graviola extracts experienced chemical changes, FTIR was used to examine solid wastes of crab, shrimp, and squid before (untreated) treatment and after interaction with GE was verified. As clearly indicated in each of Fig. 7a–c from after treatment, crab, shrimp and squid solid wastes were observed with distinct decrease in transmittance percentage. Decreased absorption means some changes (corresponding to the composition of the sample) related to bonds or phase or crystallinity;

These results suggest that graviola extracts could orchestrate (i) recovery of nanofiber's from commercial chitin material (ii) deacetylation of chitin to form chitosan and (iii) succeed in the recovery of chitosan from crude

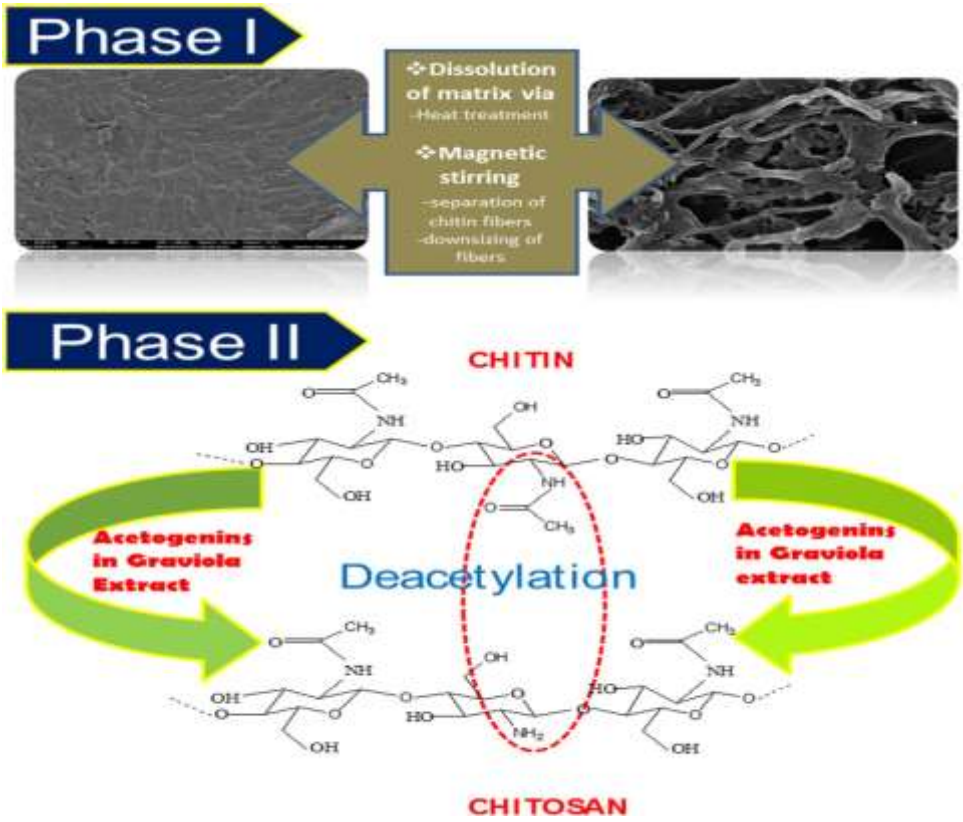

Figure8. SpeculatedmechanismorchestratedbyGEforthedeacetylationofchitintochitosan.

Chitin from solid wastes of shrimp, crab and squids. This is the first study towards that. Conventional extraction of chitin from shells consisting of stages; the first is demineralization using HCl to remove calcium carbonate, and the second step is deproteinization using a protein removal solution using NaOH to obtain chitosan a further deacetylation protocol is mandatory. The technique shown is completely green and is controlled solely by the interaction of plant extracts.

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II. DISCUSSION

The concept of using graviola extracts for chitosan recovery that were known for their anticancer activity started with the announcement of their ability to act as an insecticide. The idea of a possible interaction between the graviola extract and the chitin that conceived this pioneering work was thus brewed. The one pot, go green, protocol resulted in chitosan being reclaimed from commercial chitin as well as directly from solid marine waste. Graviola, commonly known as soursop, belongs to the family of the Annonaceae, whose scientific name is Annona muricata. Graviola plays a crucial role in various conventional and alternative medicinal products. All parts of this evergreen tree are used in natural medicine, inhabiting tropical and subtropical regions. Extracts of graviola are considered to be abundant in flavonoids, isoquinolin alkaloids and declared acetogenins51. Acetamide, acetic acid, formamide, decanoic acids and its associates, xylitol, piperidine, propionic, threonic, coumaric, xylonic, lactic, maleic acids and various bioactive sugars have been identified in the extract. The existence of alkaloids, flavonoids, terpenoids, coumarins and lactones, anthraquinones, tannins, cardiacglycosides, phenols, phytosterols, and saponins is also well established.

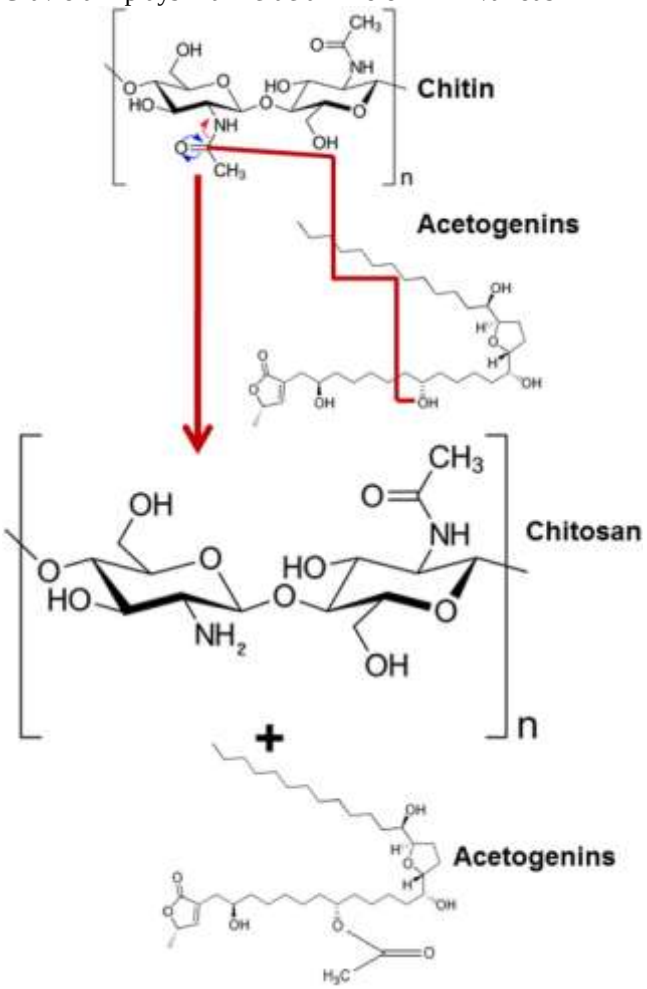

Figure9. Illustration of the acetogenin steered deacetylation reaction in the extracts of graviola.

Validate the economic importance of A. muricata leaves extracts for extensive use in medicine both traditionally and pharmaceutically.

Mechanism of action of graviola extracts.

Chitin's interaction with GE resulted in nanofibers of chitosan from industrial chitin flakes, and chitosan particles from solid shell wastes and

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squid pens. Graviola extract is a rich source of various compound types.

Graviola extracts are well-established as a source of anticancer drugs and no studies on the use of Graviola for green synthesis or phytosynthesis of nanomaterials have been reported for their bioactive potential. Figure 8 speculates the possible mode of action involved in the transition of chitin to chitosan. It is assumed that the basis for a physiochemical process is. The heating of the interactants caused the protein matrix to dissolve, releasing the chitin fibres. Magnetic stirring resulted in the fibre aggregates being separated and smoothed. The exposed fibres were now available to freely interact with the components in the graviola extract, orchestrating the deacetylation of chitin to form chitosan. In case of the solid shells and squid pens, deproteinization through heating and dissolution of chitin into the extracts and their subsequent deacetylation is the operational strategy. The chitosan recovered from the shells did not have a morphology dependent on fibre, but developed powders made from chitosan particulate. The UV Vis spectra showed that nanofibers absorbance indicated acetogenin presence. The adsorption of acetogenins on chitosan nanofibers points towards the direction of an active process for acetogenin lead deacetylation. Chitin's deacetylation reaction is possibly triggered by a nucleophilic attack of OH groups present in the acetogenins (Fig. 9).More conclusive studies will shed light on the exact modus operandi involved.

Graviola (soursop), Annona muricata L., is widely grown throughout the world's tropical regions, and it occurs as a small tree. The fruits are the edible ones, the leaves were considered meaningless before their anticancer value was revealed. With the wide abundance of this plant and with the fact that we do not use the roots or the entire plant, there is no question about the sustainable harvesting of its leaves in event of a scale-up for industrial large scale chitosan recovery. The occurrence of this plant, wild and cultivated and not endangered are additional stilts that keep the sustainability aspect strong.

III. CONCLUSION

The relationship between the graviola extracts and the chitin was examined for the first time. The findings indicated that graviola mediated one pot of chitosan recovery from chitin and solid shell wastes as hypothesised. This research pioneers the hope of a cheap and sustainable recovery of chitosan (reputed for its enormous biological applications) made possible by this green technique mediated by phyto. Furthermore, the process of extraction of graviola is achieved by itself without any chemical input, solely through a system of extraction based on hot water.

IV. METHODS

Chitin sources.

The chitin used to produce chitosan Nano fibrils was industrial crab-shell chitin, functional grade (C7170-100 G) from SIGMA-ALDRICH, Inc., 3050 Spruce Street, and Louis. MO 63103 United States 314-771-5765; the shells of crabs and shrimps and squid pens were obtained from a local restaurant in Gwanjin GU, Seoul. Südkorea. No live any In Seoul, Korea, the dried Graviola leaves used to prepare the extract were procured from a traditional medicine outlet. All chemicals used in the study were all of analytical grade, unless otherwise specified. For all tests Millipore water was used.

Graviola extract.

Four grams of dry leaves were cut into small strips (~8 leaves) and used to prepare the extracts. A method of co-boiling water extraction was practiced as our previous experiments52 showed that the method of co-boiling was better than the style of green tea (preboiling water and then adding leaves for extraction, off flame).In a jar, the leaves were applied to 200 mL of sterile water, and the contents boiled until half the original volume was evaporated. The pH extract was measured and used in the studies which followed. GE coded for the sample.

Chitosan recovery from commercial chitin.

Two grams of commercial crab shell chitin (CC) was added to 200 mL of GE and allowed to interact on a magnetic stirrer cum heater, set at 70 °C for 1, 2, 3, 4, 5, 7 days. The retrieved samples were centrifuged (Beckman Coulter Avanti J-25, USA) at 3000 rpm for 3 min to pellet the bigger debris. The pellet was discarded and the supernatant was centrifuged at 19,000 rpm for 15 min and the pellet was suspended in distilled water. One set of the pellet was dried and used for FTIR analysis.

Characterization of chitosan.

A Nano drop ND-1000 v 3.3.1 spectrophotometer (Nano drop Technologies, Inc., Wilmington, USA) was used to classify the suspended pellet following GE incubation for varying time periods. The absorbance was scanned from 220–700 nm. Fourier-transform infrared spectroscopy (FTIR) (Shimadzu FTIR-8300

spectrometer, San Diego, CA, USA) of the recovered product and of the solid wastes prior to and subsequent to treatment was done using KBr pellets. The synthesized product was imaged using field emission scanning electron microscopy (FE-SEM) (JEOL, JSM-5410LV). Using an inverted microscope, Axiovert 2000, Carl Zeiss, Germany, imaged the morphological changes on the shells and squid pens prior to and after incubation with the extract. A JEM-1400PLUS, transmission electron microscope (TEM), JEOL USA, Inc., was used to classify the chitosan obtained from contact with marine shell waste. The USA, Peabody, MA.

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