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Physicochemical and functional characterization of chitosan from horn snail gastropod *Telescopium telescopium*

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ABSTRACT

Chitosan is a naturally occurring biopolymer having immense structural possibilities to formulate novel properties and applications especially in the field of biomedical science. In view of the above, the present findings were aimed to develop chitosan from marine gastropod *Telescopium telescopium* and characterize its structural, chemical, and thermal properties by means of Fourier Transform Infrared Spectroscopy, X-ray Diffraction, Scanning Electron Microscopy and Thermogravimetric analysis. The synthesized chitosan was soluble in 1% acetic acid with a degree of deacetylation of 74.96%. The yield was estimated as 42%, with ash and moisture content of 1.2% and 3.9%, respectively, and showed binding capacities of 240% (WBC) and 280% (FBC). The synthesized chitosan exhibited good bactericidal effect towards *Bacillus subtilis* and *Vibrio cholerae*. The *T. telescopium*-derived chitosan also proves to be a novel, non-antibiotic agent preventing biofilm formation.

INTRODUCTION

Chitin is a naturally occurring polysaccharide derived from the exoskeleton or cuticle of different invertebrates such as arthropods, mollusks, cnidarians, pogonophores, and cell walls of algae (Kaya *et al*., 2014). Natural polymers receive greater attention especially in the biomedical field owing to their structural similarities with biological macromolecules that are easily metabolized into non-cytotoxic residues and naturally eliminated. Chitosan which is a deacetylated form of chitin (Azuma *et al*., 2015) possesses various biological properties like biocompatibility, biodegradability, and nontoxicity that are very advantageous in the biomedical field (Ramasamy and Shanmugam, 2015). Recently, chitosan has attracted considerable significance based on diverse novel applications.

Chitosan has received extensive attention due to its promising applications in the fields of biomedical, food, and chemical industries. The products of chitosan-based biomedical materials include hydrogel membranes, nanofibers, beads, micro and nanoparticles, and scaffolds and sponges (Jayakumar *et al*., 2010a). It is considered to be an ideal candidate to fabricate polymeric tissue scaffolds due to its high porosity, biodegradability, and structural integrity (Jayakumar *et al*., 2010b). One among the notable and much explored is its excellent antimicrobial property inhibiting the growth of a wide spectrum of target organisms like bacteria, fungi, and viruses making it beneficial for use in the field of biomedicine (Jayakumar *et al*., 2011). Microbial growth on the surface of food has been a major source of food spoilage and food-borne infections. However, chitosan possesses unique property that makes it an ideal component for the development of antimicrobial edible films and coating against these pathogens (Costa et al. 2013).

Several studies are focused on synthesizing chitosan from crustaceans (e.g., shrimp, crab, or krill shells); but incredibly limited research on gastropod-derived chitosan being reported till date. Hence an attempt has been made to utilize the shells of *Telescopium telescopium* to extract chitosan and characterize its physico-chemical, structural, and functional properties through analytical techniques.

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MATERIALS AND METHODS

Collection of *T. telescopium* shells

Telescopium telescopium commonly known as "horn snail" was utilized as a precursor to the synthesis of chitosan. The *T. telescopium* shells collected from the intertidal area of Vellar estuary (11° 29′N, 79° 46′E) were brought to the laboratory and washed thoroughly with distilled water, sun dried and ground into a fine powder.

Extraction of chitosan

The powdered sample derived from the *T. telescopium* shell was initially suspended in 5% HCl (room temperature) in the ratio of 1:15 (*w/v*) for 48 hours for demineralization, Followed by deproteinization with 3% NaOH at 70°C for 48 hours by using a solvent to solid ratio of 15:1 (*v/w*). Finally, the sample was washed several times with distilled water to eliminate the excess NaOH bound to the sample and then oven-dried at 80°C for 5 hours. The resultant product thus obtained is chitin which is further deacetylated, for the removal of acetyl group that involves treating the sample with 60% NaOH solution having a solid to solvent ratio of 1:15 (*w/v*). The residue obtained after 84 hours was washed with distilled water to neutrality and rinsed with Millipore water, which was then filtered and oven-dried at 60°C for 6 hours to obtain the final product chitosan (Sangwaranatee *et al*., 2018). For the structural and functional characterization of chitosan a standard or commercial chitosan was compared with the derived chitosan from *T. telescopium*.

Determination of yield of chitosan

The yield of chitosan was determined as the weight of the derived chitosan in relation to the weight of the powdered shell sample before treatment, by following the methodology of Nouri *et al*. (2016).

Physico-chemical properties

Solubility

The solubility of chitosan was estimated by following the methodology of Mohanasrinivasan *et al*. (2014); whereby 200 mg of extracted chitosan was added to 200 ml each of water and 1% acetic acid solution.

Determination of ash content

The ash content of chitosan was estimated by heating them in a muffle furnace which has been preheated to 600°C for 8 hours. The sample was then cooled in desiccators and weighed to obtain the ash content (Mohan *et al*., 2019).

Determination of moisture content

By employing the gravimetric method the moisture content of chitosan was determined (Mohan *et al*., 2019); wherein the sample was dehydrated in a hot air oven for 2 hours to constant weight and the variation in the weights of wet and oven-dried sample was calculated as % moisture.

Degree of deactylation

The degree of deacetylation (DD) of chitosan was estimated using a fourier transform infrared (FTIR) instrument within a frequency range of 4,000–400 cm^{-1}. The equation by Mohanasrinivasan *et al*. (2014), was used, where the absorbance at A1629.85 and A3450.65 cm^{-1} indicate absolute heights of absorption bands of amide and hydroxyl groups, respectively.

$$DD = 100 - \frac{(A1629.85\,cm^{-1} - A3450.65\,cm^{-1}) \times 100}{1.33}$$

where '1.33' denotes the ratio of A1629.85/A3450.65 for fully N-acetylated chitosan.

Functional and structural analysis

FTIR spectroscopy

The chemical structure of chitosan was achieved by using FTIR technique. The absorption spectrum for chitosan was observed in the range of 4,000 cm^{-1} to 400 cm^{-1}, using the SHIMADZU IRAffinity – 1S FTIR model spectrophotometer.

Scanning electron microscopy (SEM) with energy dispersive analysis of X-rays (EDX)

The microstructure and the elemental composition of chitosan were examined using the Scanning Electron Microscope (model: JEOL–JSM IT200) under an accelerated potential of 20.0 kV at various magnifications.

X-ray diffraction (XRD)

The degree of crystallinity of chitosan was detected using the material analyzer diffractometer equipped with Cu target X-ray tube, a monochromator filtering wave at 40 kV and 30 mA, that results in a diffraction pattern within the 20 range of 5°–80° with a scanning speed of 0.4°/minutes.

Thermogravimetric (TG/DTA)

The thermal stability of chitosan was evaluated using a Simultaneous Thermal Analyser (NETZSCH-STA 449 F3 JUPITER Instrument, Germany). A known quantity of chitosan sample was heated up to a temperature of 30–600°C with a heating range of 20°C/minutes under a nitrogen flow of 50 cm^3/minutes.

Determination of WBC

The Water Binding Capacity of chitosan was estimated using a modified method of Cho *et al*. (1998) wherein 0.5 g of chitosan was taken in centrifuge tubes, weighed, and to this 10 ml of deionized water was added. The contents were mixed well and set aside in the ambient temperature for 30 minutes with periodic shaking every 10 minutes. It was then centrifuged at 3,200 rpm for 25 minutes; the supernatant was decanted and the tubes were weighed again. The WBC was calculated using the following equation:

$$WBC\ (\%) = [\text{water bound (g)}/\ \text{sample weight (g)}] \times 100$$

Determination FBC

The Fat Binding Capacity of chitosan was estimated using a modified method of Cho *et al*. (1998) that involved weighing centrifuge tubes containing 0.5 g of sample to which 10 ml of coconut oil is added and left at ambient temperature for 30 minutes with periodic shaking every 10 minutes. It was

then centrifuged at 3,200 rpm for 25 minutes, the supernatant was decanted and the tubes were weighed again. The FBC was calculated using the following equation:

FBC (%) = [fat bound (g)/sample weight (g)] × 100.

Microbial studies

Antibacterial activity

The antimicrobial activity was carried out following the method described by Khalili *et al*. (2012), with minor modifications. The antibacterial activity for both the commercial as well as derived chitosan was tested against two pathogenic bacteria such as Bacillus subtilis (gram-positive) and Vibrio cholerae (gram-negative). Sterile disks infused in different concentrations of chitosan were air-dried and placed on agar plates and were incubated at 37ºC for 24 hours, after which the zones of inhibition were measured (in mm).

Biofilm inhibition assay

The antibiofilm efficiency of commercial as well as extracted chitosan against the Gram-positive *B. subtilis* was examined following the protocol described by Ishwarya *et al*. (2018). The bacterial inoculum was added to the culture plate having nine wells, along with a 0.1 mg/ml concentration of prepared chitosan solution. To ensure better biofilm formation the inoculated plates were incubated for 24 hours at 37°C. After the incubation period, the media was decanted using a micropipette and the formed biofilm was then fixed with 100 µl absolute methanol and further incubated for 15 minutes at 37°C. After this step, methanol was removed from the wells, and the biofilms were stained with 0.1% crystal violet and again incubated at room temperature. After 30 minutes the excess stains were washed with distilled water and the plates were left to dry for 5 hours. After drying, each well was treated with 20% glacial acetic acid solution, and this de-stained solution was evaluated for optical density measurement at 595 nm using the ELISA (Enzyme - Linked Immunosorbent Assay) reader.

RESULTS AND DISCUSSION

Yield

The total yield of chitosan contributed by *T. telescopium* was 42% (Table 1) which is comparable to the study by Palpandi *et al*. (2009), with 31.14% and 44.29% from the shell and operculum of gastropod *N. crepidularia* and Majekodunmi *et al*. (2017) with a chitosan yield of 51.8% and 43.8% from *Mytilus edulis* and *Laevicardium attenuatum,* respectively.

Table 1. Physico-chemical and functional properties of chitosan from *T. telescopium* shell.

Properties	Value
Yield	42%
DD	74.96%
Ash content	1.2%
Moisture content	3.9%
Appearance	White
Solubility	1% CH_3COOH

Ash and moisture content

Ash content is one of the vital parameters that affect solubility and viscosity. A high-quality grade of chitosan is said to have ash content less than 1%, as reported by Hong and Meyers (1995). In the present study, it was recorded as 1.2% (Table 1) which is comparable to the observation made by Vinusha and Vijaya (2019), with shrimp shell having 1.4%, fish scales with 1.8% and 1.6% from Crab shell. The moisture content of the chitosan was observed to be 3.9% (Table 1) which is in par with Majekodunmi *et al*. (2017), wherein 3.28% and 3.84% of moisture content was observed for *M. edulis* and *L. attenuatum*-derived chitosan, respectively. A report by the Korean Food and Drug Administration indicates that the moisture content of any chitosan should be <10%.

Solubility

The derived chitosan from the *T. telescopium* shell was found to be clearly soluble in 1% acetic acid and partly soluble in water. According to the literature, chitosan has been reported to be soluble in acidic media such as acetic acid, formic acid, l-glutamic acid, lactic acid, and succinic acid (Romanazzi *et al*., 2009) and feebly soluble in most common solvents, which forms the greatest limitation for scaling up the compound from laboratory to industrial level.

Degree of deacetylation (DD)

The dDD of chitosan is an important parameter influencing physical, chemical, and biological properties (Kumari *et al*., 2017). In the present study, DD of derived chitosan was found to be 74.96% (Table 1) equivalent to the observations made by Mohanasrinivasan *et al*. (2014), from the shrimp shell waste with a value of 74.82% and Kumari et al. (2017), that reported 75%, 78%, and 70% for fish, shrimp, and crab chitosan, respectively. Besides, the results by Majekodunmi *et al*. (2017) show a DD value of 69.6% from *M. edulis* and 37.3% from *L. attenuatum.* The DD greatly influences the solubility, chemical reactivity and biodegradability of the compound and varies depending on the method employed for sample preparation and the types of instruments used for the analysis (Khan, 2002). Similarly, Muñoz *et al*. (2015) reported DD of 73.6% from a fungus and stated that the value is based upon the source of chitin derivative, time, temperature, and alkaline concentration used during the process of extraction.

FTIR analysis

The functional property of chitosan was characterized by FTIR (Fig. 1). The spectrum showed a peak at 873.75 cm^{-1} that could be attributed to C–N stretching. The absorption peak at 1,454.33 cm^{-1} is due to C–H bending of the side chain $–CH_2OH$, the peak at 1,654.92 $cm^{-1}$is a characteristic bending vibration of N–H similar to the observation recorded by Kaya et al. (2014) in the bat guano indicating the formation of chitosan polymer. The band at 3,637.75 cm^{-1} is characteristic of free O-H groups, while that of 1,103.28 cm^{-1} is attributed to the C O C stretching mode that almost corresponds to the observation by Shanmugam *et al*. (2016) with 1,106.85 cm^{-1}. A high intensity of peak associated with N-H vibration indicates deacetylation of the chitin that shows

the prevalence of NH_2 groups (Majekodunmi *et al*., 2017). The spectrum is relatively equivalent to that of commercial chitosan and all these are typical absorption peaks of chitosan molecules.

SEM-EDX analysis

The structural characterization of chitosan was studied by SEM micrograph that shows the difference in the surface morphology between the gastropod derived and commercial chitosan. For *T. telescopium* chitosan the micrograph (Fig. 2a) shows a smooth and homogenous surface with clustering of uneven particles that were rod-like and disk-like structures, dispersed as flakes under high magnification, similar to the observation recorded by Masotti (2008). Yen *et al*. (2009) reported that, at higher magnification, some parts of chitosan exhibited crumbling flakes which is also observed in the present study; whereas for commercial chitosan (Fig. 2b) a non-smooth and non-homogenous surface is observed. The surface morphology of chitosan derived from different sources display different pattern, thus playing a significant role in determining application in various fields (Shanmugam *et al*., 2016). The elemental composition of the chitosan was studied by EDX that showed the presence of carbon, oxygen, calcium, zinc, iron and magnesium elements.

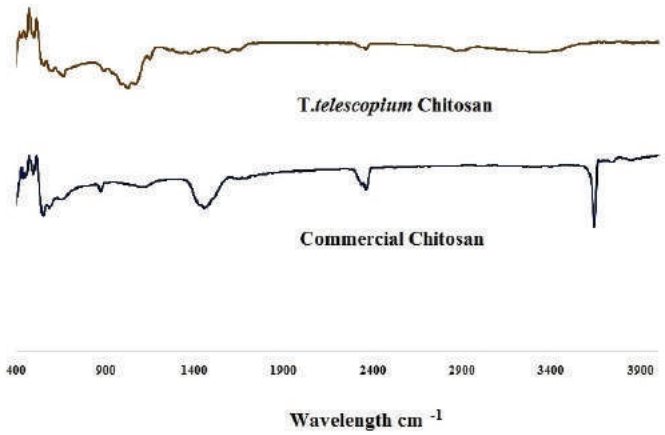

Figure 1. FTIR spectra of *T. telescopium* chitosan and commercial chitosan.

XRD analysis

The XRD pattern of chitosan (Fig. 3) exhibited a significant peak at 2θ = 9.9–10.9º and sharp crystalline reflections at 20 = 18–20º and around 34–35º which are typical finger prints of semi-crystalline chitosan similar to the XRD pattern derived from the marine crab shell chitosan (Munusamy *et al*., 2017). Similarly, Yen *et al*. (2007) found that fungal chitosan displayed two crystalline reflections at 9.7º and 19.9º; while Harish Prashanth *et al*. (2002) recorded two major characteristic peaks for shrimp chitosan at 20 = 9.9−10.7º and 19.8–20.7º. A study by Yen *et al*. (2009)

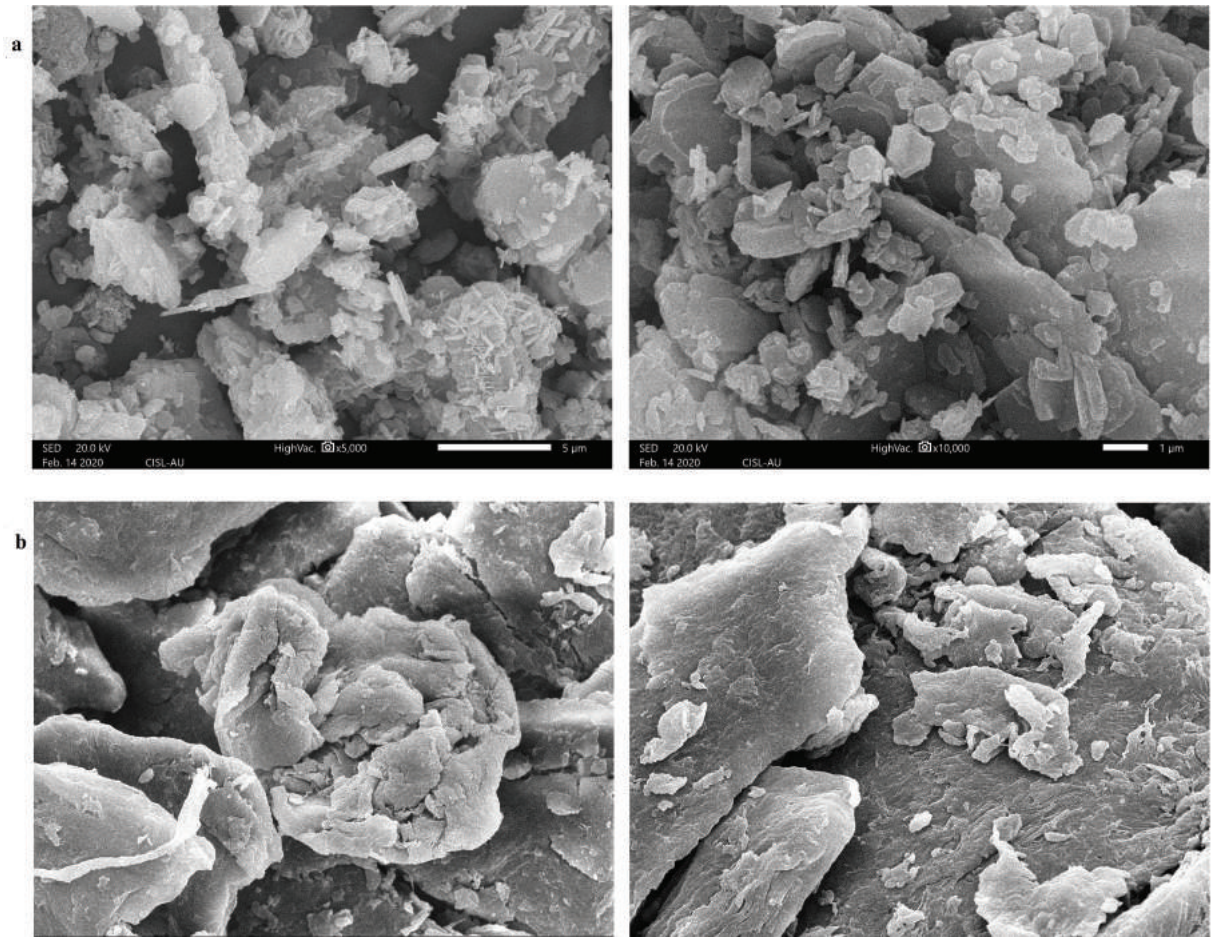

Figure 2. SEM micrograph of (a) *T. telescopium* chitosan and (b) commercial chitosan.

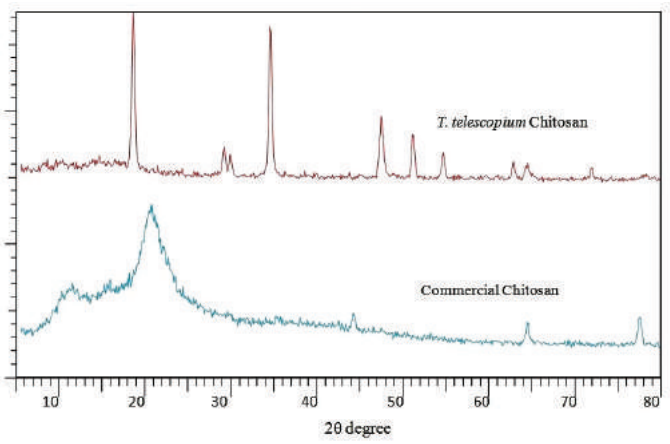

Figure 3. XRD pattern of *T. telescopium* chitosan and commercial chitosan.

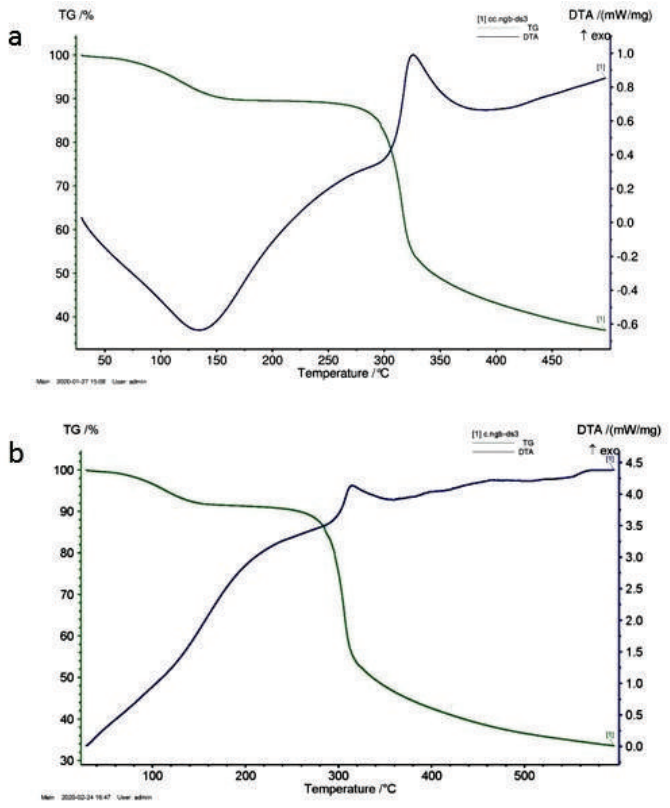

Figure 4. TG/DTA curves for (a) *T. telescopium* chitosan and (b) commercial chitosan.

reported two characteristic peaks (α- and γ-chitosan) with slightly fluctuated diffraction angles, exhibited an equivalent degree of crystallinity with peaks formed at 18–19º and 34–35º respectively. The diffraction pattern of commercial and extracted chitosan shows more or less comparable intensities of peaks, which shows the crystalline and amorphous properties of the compound.

TG/DTA

The TG/DTA curves of the derived chitosan and commercial chitosan recorded in the temperature range of 0°C−600°C are shown in Figure 4. The TG curve of chitosan displays two steps of decomposition. The first step of degradation due to release of typical strong hydrogen-bonded water (Zawadzki and Kaczmarck, 2010) was observed at 104°C and 113ºC for derived and commercial chitosan, respectively. Subsequently, the second step, which is the degradation of saccharide structure in the molecule, was recorded as 324ºC and 313ºC for derived and commercial chitosan respectively. The maximum thermal degradation was reported as 444°C and 438ºC for the prepared and commercial chitosan, respectively; which shows that extracted chitosan is more stable compared to the commercial chitosan molecule. De Andrade et al. (2012) has reported a maximum decomposition range of 400°C–500°C for crab chitosan.

WBC and FBC

Water and Fat binding capacities of chitosan are functional properties that vary based on the technique followed. The derived chitosan has a WBC of 240% which is less compared to previous studies by Kumari *et al*. (2017) with a WBC of 358% for shrimp shell chitosan. Similarly, No *et al*. (2000) reported WBC of five commercial chitosans that ranged between 355% and 611%. Knorr (1982) illustrated that variation in WBC mainly depends upon the source of chitosan, variation in crystallinity, number of salt-forming groups, and residual protein content of chitosan. The above-mentioned parameters could be the reason for the reduced WBC value of the prepared chitosan. The FBC of the prepared chitosan was 280% which is comparable to the observations made by Kumari *et al*. (2017) with 246% from shrimp shells and No *et al*. (2000) that reported 217%–403% from five commercial chitosans.

Antibacterial activity

The minimum bactericidal concentration (MBC) of chitosan was studied for two bacterial strains. At 10, 20, and 30 mg/ml concentrations of chitosan, a slight effect on the growth of *B. subtilis* was observed. However, at 40 mg/ml concentration, the growth of *B. subtilis* was repressed with a 0.2 cm zone of inhibition for both the synthesized and commercial chitosan; furthermore, at 50 mg/ml concentration the zone of inhibition was observed at 0.4 cm for both chitosan compounds. This shows a decrease in the concentration of chitosan may lead to a decrease in the zone of inhibition. For *V. cholerae* a partial zone of inhibition was observed at all concentrations for synthesized chitosan, while commercial chitosan showed 0.6 cm inhibition at a concentration of 40 mg/ml and slight zones for the rest of the concentrations. Generally, the microbial inhibitory activity of chitosan is based on its chemical and structural properties (Raafat and Sahl, 2009). The present investigation reveals that synthesized chitosan showed good inhibitory activity similar to the commercial chitosan towards both pathogenic bacterial strains, *B. subtilis* and *V. cholerae.* Study by Qin *et al*. (2006) described that chitosan with low-to-medium molecular weight and having a high DD of over 80% is observed to restrain the development of both gram-positive and gram-negative bacteria.

Biofilm inhibition assay

The effect of chitosan on preventing biofilm formation has been studied by various researchers (Rinaudo, 2006) and is

now been widely accepted that the biofilm is the predominant mode of bacterial growth that differ substantially in terms of growth rate, gene expression, and structural properties (Donlan and Costerton, 2002). Hence, testing for antimicrobial properties not only against growing bacterial colonies but also against microbial biofilms is mandatory. During the present study only a single concentration of 40 mg/ml chitosan extract was tested against biofilm formation in all the nine wells for *B. subtilis*; as the MBC was best observed at this minimal concentration of chitosan. A 32% and 68% reduction in biofilm formation against the grampositive bacteria (*B. subtilis*) was obtained from the derived and commercial chitosan respectively; which is indicative of antibiofilm mode of action (Batoni *et al*., 2016). Hence the biofilm-preventing activity of the synthesized chitosan also promises to be a simple and practical agent for biofilm control in the fields of food and medical industry.

CONCLUSION

Recently several health care, biomedical, and pharmaceutical industries are in need of high quality, biocompatible and biodegradable substances like chitosan for addressing many health related issues. Fortunately huge quantities of gastropod shell wastes are available as biowastes around the coastline that could be transformed as a potent source of chitosan. By following this aspect chitosan was synthesized using *T. telescopium* shell waste which is an abundant raw material along Vellar estuary and screened for its structural and functional properties using Fourier Transform Infrared Spectroscopy, XRD, TG/DTA, and SEM analysis. The good quality yield contributed by *T. telescopium,* a high DD and antimicrobial properties indicate that *T. telescopium* is a potential natural source of chitosan that can perform as a promising ingredient in the research and development of novel biomedical products.

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AUTHORS CONTRIBUTION

Concept, Data acquisition, Data analysis / interpretation and Drafting manuscript by Miss. T. Jebarani Rajathy; Designing, Critical revision of manuscript, Statistical analysis, technical or material support by Dr. T. Mohanraj; Investigation and Supervision by Prof.M.Srinivasan

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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