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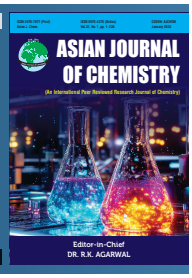

Green Synthesis of Chitosan from *Aspergillus niger* and Evaluation of Antioxidant Property and Cytotoxic Effects on Selected Cancer Cell Lines

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Chitin and chitosan, derived from fungal cell walls, possess significant biomedical importance due to their unique material properties. Chitin and chitosan were extracted from *Aspergillus niger* strains (BSH5 and BSH9) using a two-phase extraction method. The biopolymers were characterized by FTIR, X-ray diffraction (XRD), ^{1}H NMR spectroscopy and scanning electron microscopy (SEM). The thermal stability of chitin and chitosan was studied using thermogravimetric/differential scanning calorimetry (TGA-DSC). Fourier transform infrared (FTIR) was used to analyze the functional groups and validate the composition of the materials. The broad absorption band corresponding to the hydrogen-bonded O-H absorption band overlapped with the N-H band. ^{1}H NMR also confirmed the purity of the biopolymers. X-ray diffraction (XRD) patterns of chitin and chitosan show the semi-crystallinity, a potential layered crystalline structure and a similar pattern to the commercial polymers. TGA-DSC analyses demonstrated the thermal stabilities of chitin and chitosan. Structural morphology by scanning electron microscopy (SEM) revealed thick and rough surfaces indicating the presence of amorphous nature of the material. Antioxidant studies revealed the radical scavenging activity of 46.38% and 47.65% for strains BSH5 and BSH9, respectively. Chitosan from both the stains demonstrated significant cytotoxic effect against MCF7 cell line with IC_{50} values of 206.6 µg/mL and 244.7 µg/mL, respectively. The material characterization and biological attributes demonstrated potential biomedical applications of the prepared biopolymer chitosan.

Keywords: Chitin, Chitosan, *Aspergillus niger*, DPPH activity, MTT assay.

INTRODUCTION

Chitin is one of the most abundant natural polymers, second only to cellulose [1]; nonetheless, chitosan is more often used than chitin for various commercial applications [2,3]. Chitosan is acid-soluble and more easily malleable than chitin. Chitosan is a randomly distributed linear, hetero-polysaccharide composed of 2-acetamido-2-deoxy-D-glucopyranose and 2-amino-2-deoxy-D-glucopyranose units. Chitin, a crustacean byproduct from the industrial seafood processing, is chemically deacetylated to chitosan. The deacetylation is performed using a strong alkali [4]. Initially, chitin and chitosan were obtained from marine organisms, molluscs, crustaceans, shrimps, crab shells and marine invertebrates [5,6]. However, the limited supply and seasonal availability of crustaceans significantly impact the procedure. In addition, a major disadvantage of the process is that it generates a large amount of concentrated alkaline solution waste, which contributes significantly to environmental pollution. A solution to these problems is the production of chitosan from fungi due to the absence of allergenic compounds and less waste, which opened the door for further research and biotechnological applications [7]. Moreover, fungi-derived chitin and chitosan would provide consistent product characteristics and reliable, non-seasonal sources of raw materials [8]. Thus, the yields and physico-chemical qualities of chitosan extracted from a fungal organism can be improved by modifying the production through fermentation process and related conditions.

Fungal mycelium is a root-like structure of a fungus composed of a mass of branching, filamentous hyphae. Regardless of location and season, it is possible to obtain mycelium through simple fermentation [9]. Chitin is found in the cell walls of fungi,

in species in their spores and hyphae. However, the fungal cell wall consists of 2-42 % chitin [10]. Chitin forms an amorphous matrix of glucan molecules in the form of micro-fibrils [11]. Chitosan is found in some fungi belonging to the Zygomycetes class such as *Mucor*, *Absidia*, *Rhizopus* sp., *etc.* and consists of chitosan as a structural component in their cell wall [12]. The chitosan content in animals and fungi depends not only on the species but also on the age and environmental conditions of the organisms. The content in the dry shells of crab, shrimp and lobster is 14-27% [13], while the fungal cell wall consists of 2-42%. The lowest value corresponds to yeast and the highest to zygomycetes [14]. Due of its species-dependent nature, fungal chitin and chitosan production from fungal species has received more attention than from crustacean sources.

Chitin and chitosan are the basic polysaccharides, unlike other biopolymers like cellulose, dextrin, pectin, agar, alginic acid, *etc.* which are acidic. They are readily soluble in many aqueous acidic and mineral solutions, contributing various properties such as metal chelation, viscosity, biofilm formation, polyelectrolyte formation, optical characteristics, polyoxy salt formation and several other structural characteristics [15]. Since chitosan is a straight-chain polyamine, it has highly active amino and hydroxyl groups and chelates a greater number of transition metal ions. Many biological properties involve a safe, non-toxic and biocompatible natural polymer that may be biodegradable to standard body components. It effectively regenerates connective gum tissues. It binds aggressively to the surface of most mammalian and microbial cells. It increases bone formation through osteoblast production and depresses the central nervous system. It has an antitumor, spermicidal, fungi static, haemostatic, anti-cholesteric and immune adjuvant function [16,17].

The versatile molecule chitosan finds its role in various application studies. Due to its poor solubility, chitin finds limited use in application studies, however, modified chitin and chitin derivatives are used as raw materials to produce the synthetic polymer [17]. Synthetic fibres from these biopolymers are used as wound dressing elements and absorbable structures [18]. These fibres are said to accelerate wound healing by 75% [19]. These materials fight the attack of bile, urine and pancreatic juice, while other fibres are less effective. Besides medicinal use, chitin and chitosan derivatives are widely used in wastewater treatment by chelation to remove heavy metal ions [19].

Chitosan-blended antibacterial films were synthesized by irradiating the starch-based mixture, which was compressed into a gel with an electron beam accelerator at room temperature [20]. The environment in tensile strength and flexibility of the film was achieved by incorporating 20% chitosan into the starch film.The current study emphasizes on the isolation of potential fungal strains from their natural habitat for elevated production of chitin and chitosan. Conventional methodology for chitin and chitosan production from fungi was modified to achieve an economical, eco-friendly and sustainable production of these biopolymers. Material characterization of such biopolymers was performed to confirm the material nature and identify material properties, degree of deacetylation (DD%), molecular weight (MW), *etc.* Using these properties, fungal chitosan was chosen for an extensive biological study. For instance, chitosan with low MW and high DD% was considered for antioxidant and cytotoxic studies.

EXPERIMENTAL

Standard chitin from Hi-Media Labs Pvt. Ltd and chitosan from the crustacean source were purchased from Sisco Research Laboratories Pvt. Ltd., Mumbai, India. Cetyltrimethylammonium bromide (CTAB, 2% w/v), 100 mM Tris HNO_3, pH 8.0, 20 mM EDTA disodium (Na_2EDTA), 1.4 M NaCl and β-mercaptoethanol were added to 1% final volume immediately before use. The culture medium used was standard buffered Sabouraud Dextrose Broth (SDB) and Agar (SDA). The media comprised of 4% w/v dextrose, 1% peptone, 2% agar (pH 5.6).

Isolation and identification of fungal strains from soil and fruit samples: The dilution plate technique was used to isolate the fungal strains from soil and fruit samples. The rhizosphere part was employed for the collection of soil. Meanwhile, fruits with higher sugar content were selected and divided into healthy and rotten fruits. The soil/fruit preparation was streaked on sabouraud dextrose agar (SDA) plates and incubated at 28 ºC for 5 days [21]. Colony morphology was studied and phenotypic classification was done. In addition, the fungal isolates were identified by nuclear ribosomal DNA ITS sequencing [22]. DNA extraction from fungal isolates was performed according to the standard protocol [22]. The isolated DNA was quantified using 0.8% agarose gel electrophoresis (AGE). The prepared DNA was quantified using a UV-Visible spectrophotometer (Vivaspec Bio-Photometer, Germany, SMB80). The purity of DNA preparation was recorded as A_{260}/A_{280}. A PCR amplification of the ITS region was performed. A known volume (20 µL) of PCR reaction mixture containing Taq DNA polymerase buffer, 1X (Kappa, SA); $MgCl_2$, 3 mM; dNTP mix, 0.25 mM; *Taq* DNA polymerase, 0.05 U; Primers, 1 picomole and template DNA, 50 mg were added. Nuclease-free water was used as a negative control [22].

Preparation of chitin and chitosan from fungal biomass by two-phase extraction method: After growth, the fungal mycelium (*Aspergillus niger)* was filtered using Whatman filter paper grade no. 41 and dried in a hot air oven at 50 ºC for 10-12 h. Chitin and chitosan were extracted using a two-phase extraction technique. Dried fungal biomass was pre-treated by soaking in 1 N NaOH for 24 h and then de-alkylated with 0.1 N NaOH solution before being deacetylated with sulphuric acid (1%). A known amount of fungal biomass (1 g dried) was treated with 0.1 N NaOH (50 mL) before homogenization and sterilization. The mixture was then centrifuged for 10 min at 8,000 rpm. The biomass pellet was harvested, rinsed several times with sterile water until neutral pH was achieved and then dried at 50 ºC. The dried fungal biomass pellet is now known to be alkali-insoluble fraction after being sterilized and treated with sulphuric acid (1%). The acid-insoluble contents were collected by centrifugation for 20 min at 8,000 rpm. The supernatant was pooled and cleared by filtration using Whatman filter paper No. 41 on an ice bath. The solution was centrifuged for 15 min at 6,000 rpm to remove the chitosan. Chitin and purified chitosan were analyzed with the minor modifications according to the reported method [11]. Dilute sulphuric acid

was used rather than dilute acetic acid for the deacetylation process to assist the revival of chitin and chitosan. The novel approach facilitates the requirement of delayed digestion time at high temperatures.

Characterization of chitin and chitosan: Chitin and chitosan were characterized by FTIR spectroscopy (NICOLET FT-IR 6700) documented in KBr pellets in the wavelength range of 4000-400 cm^{-1}. In addition, other characteristic parameters such as degree of deacetylation (DD), molecular weight (MW) and viscosity (η) for chitin and purified chitosan were studied with FTIR spectroscopy [12] and the absorbance mode was used to measure an absorption ratio of A_{1655} and A_{3450} using eqn. 1:

$$DD\ (\%) = 100 - 75.19\frac{A_{1655}}{A_{3450}} \quad (1)$$

where A_{1655} is the absorbance of amide I band and A_{3450} corresponds to –OH groups. The molecular weight obtained was calculated by the Mark-Houwink equation (eqn. 2).

$$\eta = KMa \quad (2)$$

where η = intrinsic viscosity; K and a are the constants based on the extent of deacetylation of chitin and purified chitosan. The Ostwald's viscometer method determined the viscosity of chitin and chitosan [13]. Chitin (1% v/v) and purified chitosan (1% w/v) solutions were prepared in acetic acid and mixed for 4 h, followed by viscosity measurement at 25 ºC. The following eqn. 3 was used to calculate the viscosity of chitin and purified chitosan:

$$\eta = \left(\frac{\eta_{sp}}{C}\right) \quad (3)$$

where η is intrinsic viscosity, η_{sp} is specific viscosity and C is the material concentration.

Chitin and chitosan samples were further studied by X-ray diffraction (XRD, Bruker D8-Advance) facilitated with Cu*K*α radiation (λ = 1.54 Å) and Ni-filter with a relative intensity range of 10-90º with a scan rate of 5º min^{-1}. ^1H NMR spectroscopy was carried out with a BRUKER (400 MHz) NMR spectrophotometer. Thermogravimetric (TGA/DSC) analysis was performed using an 851^e Mettler Toledo instrument. The samples were detected under an nitrogen environment with a temperature rate of 10 ºC min^{-1}. SEM (FEI Sirion XL30 FEG SEM) was used to examine the surface morphology of chitin and purified chitosan. For SEM analysis, the samples were layered with thin coats of gold-palladium using a sputter coater apparatus and the cross-sectional landscape was analyzed with a stereo scan scanning electron microscope (440 Quanta FEG-250).

Antioxidant activity: The DPPH free radical scavenging assay has been employed to determine the antioxidant properties of chitosan. The reference was standard chitosan. The DPPH radical-scavenging activity of chitosan was analyzed by adopting the reported method [23]. A 1 mL of chitosan prepared in 0.5% acetic acid solution, with a concentration range (0.1-1.0 mg/mL), 1 mL ethanol and 1 mL of 0.1 mM DPPH solution was prepared to 4 mL final volume with 0.5% acetic acid. The reaction contents were mixed and then incubated at the room temperature for 30 min in a dark environment. Optical density readings were taken at 517 nm against a suitable blank (without DPPH) using a double-beam UV-Vis spectrophotometer (Lab India, UV-3000). Butylated hydroxy anisole (BHA) was included as a positive control and the antioxidant (DPPH scavenging) activity was calculated using the eqn. 4:

$$\text{DPPH radical scavenging activity } (\%) = \frac{A_{control} - A_{sample}}{A_{control}} \times 100 \quad (4)$$

where $A_{control}$ is the absorption value for control and A_{sample} is the absorption value for sample, respectively.

Cytotoxic activity: The MTT assay was used to analyze the cytotoxic activity of chitosan. The breast cancer (MCF7) cell line, the lung cancer (A549) cell line and the leukaemia cancer (K562) cell line were used and the degree of cytotoxicity was estimated [24]. The cells were seeded at a cell-density of 5×10^3 cells/well in a 96-well microplate and incubated overnight at 37 ºC, 5% CO_2 in 95% humidity. A sample with different concentrations (800, 400, 200, 100, 50, 25 µg/mL) was treated. Incubation of the cells was continued for another 48 h. The cells were washed with phosphate buffer and 20 µL of the MTT reagent (5 mg/mL) was added, followed by incubation at 37 ºC. Approximately 100 µL of DMSO was added after 4 h to each well. Thus, dissolved formazan crystals were estimated by reading the absorbance at 570 nm using a microplate reader. The following formula calculated the cell survival rate:

$$\text{Surviving cells } (\%) = \frac{\text{Mean OD of test compound}}{\text{Mean OD of negative control}} \times 100 \quad (5)$$

RESULTS AND DISCUSSION

Molecular identification and preparation of chitin and chitosan from *Aspergillus niger* strains (BSH5 & BSH9): Isolation of fungi from soil samples yielded *A. niger* (BSH5). The rhizosphere of the soil was exploited to isolate these fungi. The colony morphology of each fungal strain was recorded as part of phylogeny studies. Isolation of fungi from fruit samples yielded *A. niger* (BSH9). Two different *A. niger* strains were isolated with different molecular properties and biological activity. The molecular tool, ITS rDNA sequencing, was used to perform molecular identification of isolated fungi. BSH5 and BSH9 samples are amplified using ITS 4 and ITS 6 primers (PCR products; 550-650 bp). Both organisms were found to be *A. niger* of gene length 599 bp and 579 bp with 100% similarity. The sequence information was submitted to the GenBank and received the respective accession numbers as OQ236103 for *A. niger* (BSH5) and OQ236235 for *A. niger* (BSH9), respectively. Chitin and chitosan yields from fungal strains were recorded in terms of dry weight (w/w). *A. niger* (BSH9) strain has shown the highest yield of chitin and chitosan (0.4479 g/L chitin and 0.2351 g/L chitosan). Whereas *A. niger* (BSH5) strain has shown lesser quantity of chitin (0.1755 g/L) and chitosan (0.1424 g/L). The two-phase extraction procedure for fungal chitin and purified chitosan was slightly tailored for the following reasons. When using acetic acid or HCl to extract chitosan, a low-yield product with high phosphate impurity was obtained [25]. Recently, the treatment of fungal cell walls with a hot

dilute sulphuric acid solution has led to a high yield and more purity of chitosan. Unlike acetic, citric, lactic and HCl, chitosan is insoluble in dilute H_2SO_4 solution at room temperature. The solubility of chitosan in hot H_2SO_4 medium is the temperature dependent, which is not shared by any other components of such as chitin and polyphosphates. Therefore, by treating the cell wall with hot dilute H_2SO_4, chitosan becomes soluble in hot acid and it can be separated from other cell wall components by filtration, which can be recovered easily by cooling.

Characterization studies for chitin and chitosan

FTIR studies: The characterization of fungal chitin and purified chitosan was carried out by FTIR analysis to validate the extracted material. The broad spectral bands of fungal chitin (Fig. 1A) show the OH-axial stretching between 2922.77 cm^{-1} and 3430.15 cm^{-1}, superimposed on the NH stretching band, with the axial deformation of amide C=O at about 2372.20 cm^{-1}, whereas the angular deformation of –NH at 1638.00 cm^{-1} and the axial deformation of amide –CN at 1429.54 cm^{-1} are also observed. Moreover, the symmetrical angular deformation in CH_3 is oberved at 1377 cm^{-1} and the –CN axial deformation of amino groups occured at the 1162.65-1115.29 cm^{-1} range. The structure bands of polysaccharides in the region between 1024.92-580.75 cm^{-1}. Therefore, the FTIR spectral pattern of the purified chitin prepared from the fungal strains is consistent with the literature [26]. In addition, FTIR spectrum of commercial/standard chitin are also given for comparison (Fig. 1A). Compared to the commercial/standard chitin, almost similar functional groups are present in the fungal chitin, confirming the purity of the sample. The method of analyzing the results was similar for chitosan. Still, differences in the measured values and the band stretching of each chemical functional group and hence the configuration also varies accordingly.

FTIR studies of purified chitosan (Fig. 1B) show key bands between 3800-350 cm^{-1}. The broadband associated with the OH axial stretching between 3729.68-3432 cm^{-1} superimposed on the NH stretching band was observed. The peak at 2927 cm^{-1} showed an asymmetric CH_2 stretching, whereas an aliphatic CH stretching was observed at 2850 cm^{-1} in chitosan. With the axial deformation of amide C=O at 1632.03 cm^{-1} and the angular deformation of NH at 1559.11 cm^{-1} is observed. Similarly, the axial deformation of amide (–CN) appeared at 1464.90 cm^{-1}, the symmetrical angular deformation in CH_3 at 1376 cm^{-1} and the –CN axial deformation of amino groups in 1158-1223 cm^{-1} range are observed. Finally, the polysaccharide structure bands occurred in the region between 1120-876 cm^{-1}. The results of the present study were similar to those observed by other researchers [27-31].

In another study, the alkaline soluble and insoluble glucan and chitosan showed spectral peaks at 1550 cm^{-1}, an amide II band which is very near to the IR spectra values of the fungal chitosan extracted from standard and isolated fungi. The results showed that few chitosan molecules are entrapped in glucan triple helices, where the heat-stable α-amylase enzyme also cannot show cleavage activity [32]. Since the FTIR analysis of all the spectral bands is consistent with the commercial/standard chitosan, the nature of the extracted material and some other molecular characteristics such as molecular weight (MW), degree of deacetylation (DD%) and viscosity (cP) were studied. The absorption spectra by FTIR were taken for the calculation of the DD% and other molecular characteristics such as molecular weight and viscosity and are presented in Table-1, which shows that DD% of fungal chitin is lower than that of chitosan. Standard chitin and chitosan from crustacean sources have a higher DD% than chitin and chitosan from fungi. However, commercial chitosan has 88.08%.

TABLE-1
MOLECULAR PROPERTIES OF STANDARD CHITOSAN AND CHITOSAN OBTAINED FROM *A. niger* (BSH5 AND BSH9)

Material	Degree of deacetylation (DDA %)	Molecular weight (MW)
Chitosan (standard)	88.08	1.2×10^6 Da
Chitosan (BSH5)	79.08	2.1×10^5 Da
Chitosan (BSH9)	79.33	4.9×10^6 Da

NMR studies: Typically prepared chitosan (BSH5) from fungi was characterized by ^1H NMR spectroscopy to know the purity of the isolated chitosan and to determine the degree

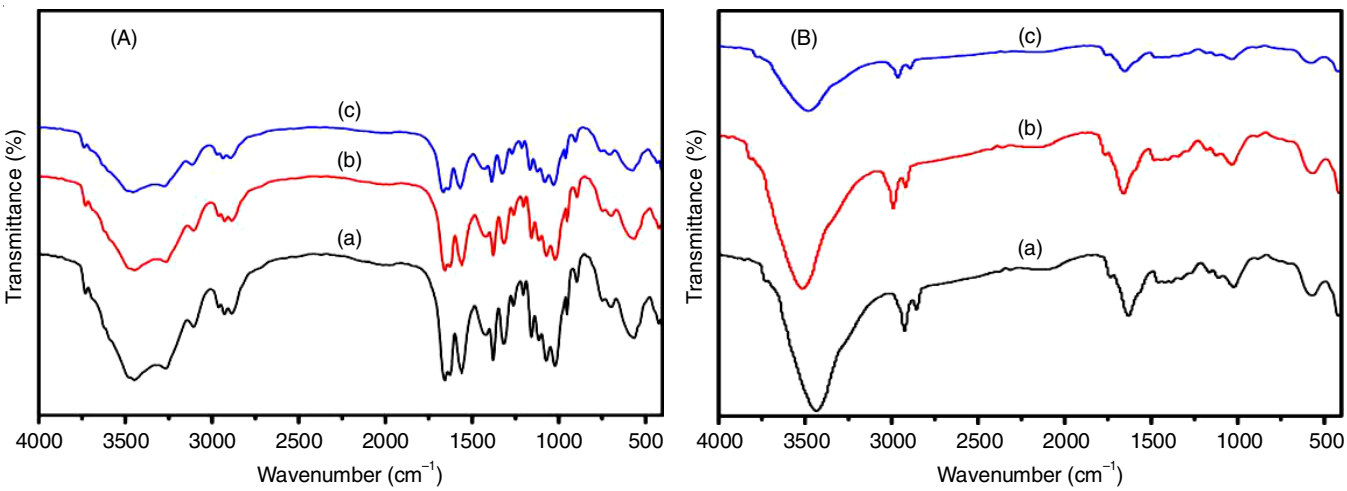

Fig. 1. FTIR spectra of chitin (A) and chitosan (B); (a) standard chitin/chitosan, (b) chitin/chitosan from *Aspergillus niger* BSH5 and (c) chitin/chitosan from *Aspergillus niger* BSH9

of deacetylation (DD). It was found that the DD extent from the -CH_3 group and hydrogen H-1 GlcNAc decreases as the molecular content of N-acetylglucosamine in the chitosan decreases. The spectra results confirm the successful alteration of Cys on the chitosan chain. ^{1}H NMR spectra of extracted chitosan demonstrated acetyl protons at δ 1.6 ppm. The spectra of purified chitosan be likely to show acetyl protons at δ 2.1 ppm where resonated at δ 3.2 ppm, which is due to internal deacetylation of H-2 [33]. The H3-6 (ring) and H-2 (acetylated units) showed at δ 3.5 and 4.1 ppm, respectively (Fig. 2), which corresponds to the level of standard chitosan. The H-1 from deacetylated components and OH resonate likewise as reported earlier [34]. NMR analysis also established the 83.15% deacetylation which is corroborated by the FTIR results.

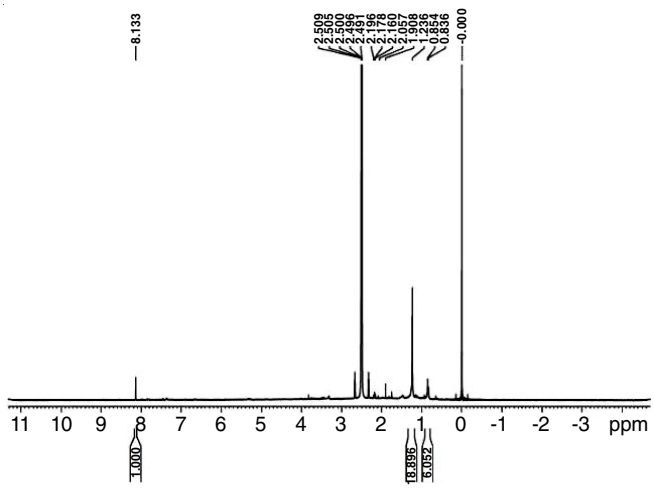

Fig. 2. ^{1}H NMR spectra of chitosan obtained from *Aspergillus niger* BSH5

XRD studies: Fig. 3 shows the powder XRD pattern of a commercial sample, chitin and purified chitosan extracted from *A. niger* to determine the crystallinity. Chitin and purified chitosan extracted from fungi showed a sharp peak between 10-40º, with the strongest peak at 2θ = 20.91º in both BSH5 and BSH9 samples. However, similar peaks were obtained for commercially obtained chitin and/or chitosan samples used for the comparison purposes. The characteristic peak at 20.91º obtained for both chitin and chitosan (BSH5) in the current study confirm a dense crystalline structure [35]. Moreover, the broad peaks are also observed in the XRD pattern of chitin and chitosan prepared from fungi (BSH9), which confirms that the obtained materials are more amorphous, similar to the earlier studies [35].

An additional study reported that the greater reflection for purified chitosan at around 30-35º is almost identical as observed in the present study [35]. Some authors have reported characteristic diffraction peaks at different intensities [36,37]. The XRD results show that the crystallinity of the membranes depends on the polymer concentration used for the study. In present study, pure chitin and chitosan exhibit a crystalline nature for the BSH-5 and an amorphous nature for the BSH-9 sample [35]. It is clear from the XRD that chitin and chitosan were extracted from the isolated fungi.

SEM studies: The surface and internal morphologies of chitin and purified chitosan are shown in Fig. 4. SEM analysis was performed at 5000X magnification and the samples showed bulky and irregular surface properties at this magnification, confirming the amorphous and/or semi-crystalline nature of the biological extracted material [35]. It is also observed that chitosan (BSH5) extracted from *A. niger* has a distinct nearly spherical structure with a network of greater porosity and a structure of smaller dimensions (a narrow size distribution). However, in case of BSH9, chitosan has a fibre-like structure with numerous pores. As one can observed that chitosan is more crystalline than chitin, which is also confirmed by XRD analysis. These results are similar to previous studies on BSF pupal shell-derived chitin and chitosan reported [38], where the surface properties revealed a compactly packed structure

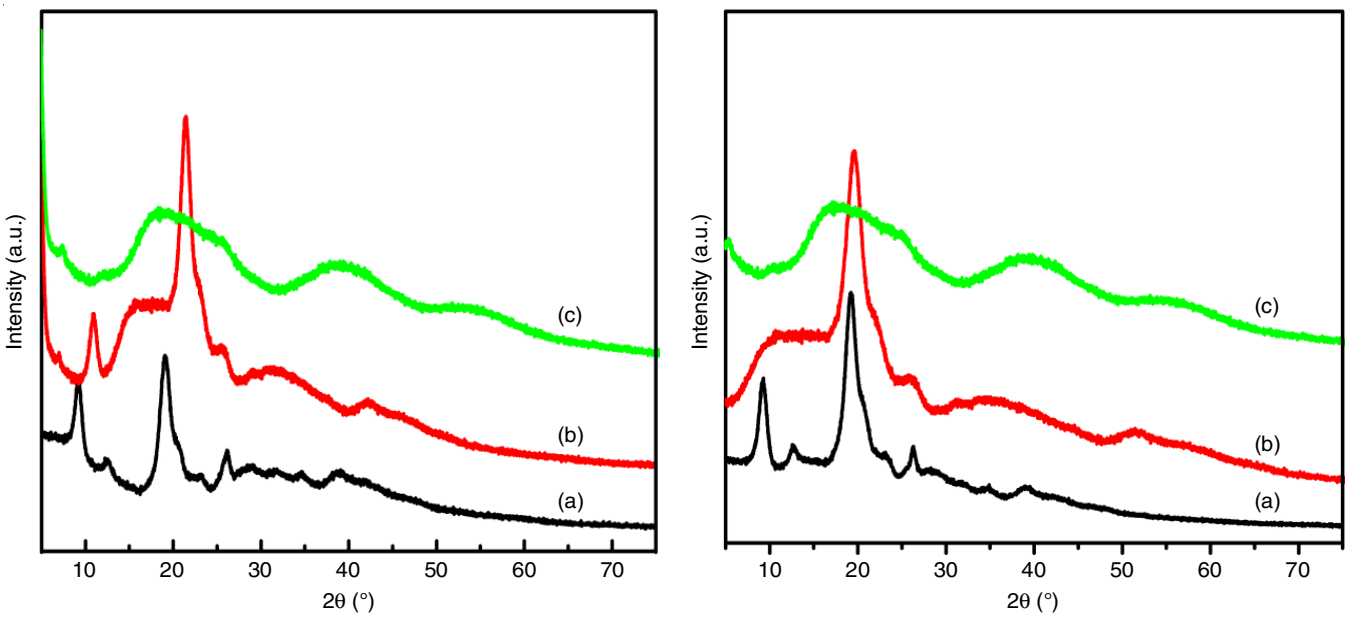

Fig. 3. XRD pattern: (a) standard chitin/chitosan, (b) chitin/chitosan from *Aspergillus niger* (BSH5) and (c) chitin/chitosan from *Aspergillus niger* (BSH9)

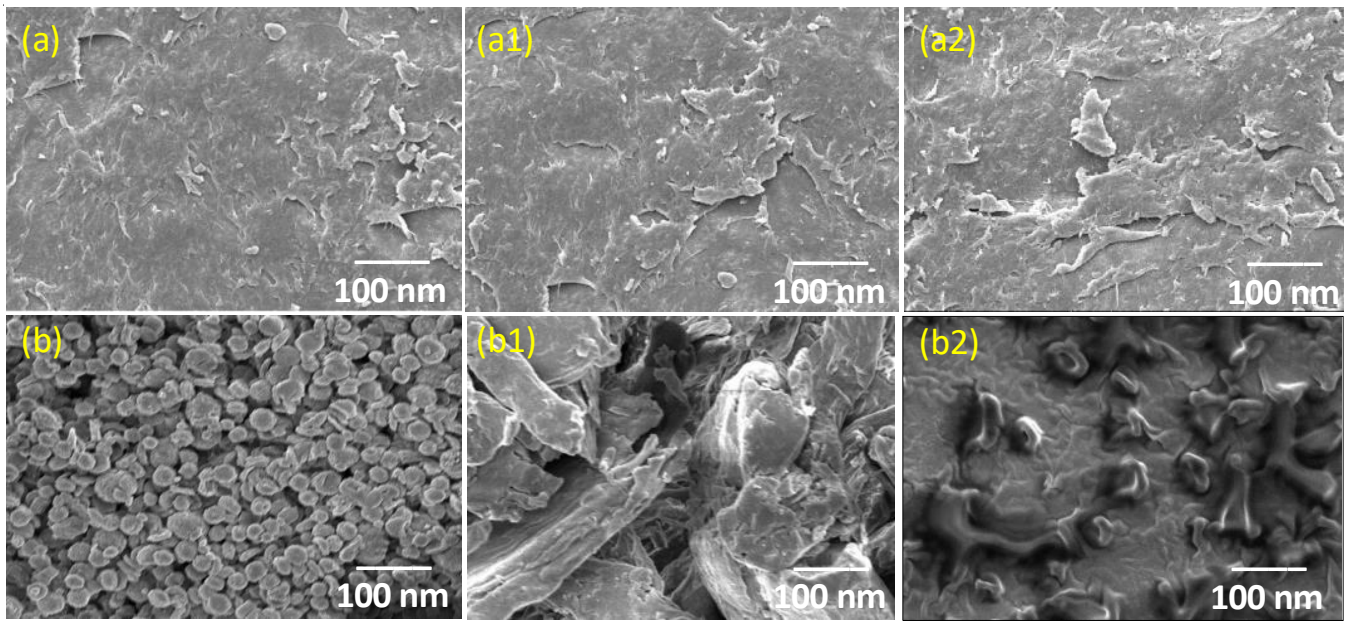

Fig. 4. SEM images of (a) chitin *A. niger* (BSH5), (b) chitosan *A. niger* (BSH5), (a1) chitin *A. niger* (BSH9), (b1) chitosan *A.niger* (BSH9), (a2) chitin (standard) and (b2) chitosan (standard)

with repeating hexagonal and circular units. An irregular surface with fibres and pores with a 20 µM diameter was observed. According to the previous literature [39], chitin and chitosan prepared from different sources have different morphologies. The surface properties of the biologically obtained chitin and chitosan from the *A. niger* in the current study is same as compared to other organisms, such as marine crabs and grasshoppers [40].

Thermal studies: From Fig. 5a, it can be observed that chitin extracted from fungi had a water loss of 60 ºC and degradation happens in the 250-380 ºC temperature range, while chitosan prepared from fungi has a water loss at 80 ºC and degradation occured within 300-400 ºC range. The results are similar to those of earlier studies [34]. The weight loss in the temperature range of 60-120 ºC is in all cases attributed to the loss of absorbed water. The second weight loss in the temperature range around 250-400 ºC corresponds to the chemical disintegration and deacetylation of prepared chitin and purified chitosan [34]. Furthermore, the later step includes the oxidative degradation of the carbon residue produced in the earlier step in the temperature range of 400–600 ºC. The thermal stability of chitosan is slightly higher than that of chitin, which is observed in thermal analysis in the form of water loss in chitin. With the reduction in the molecular weight, more water loss is observed during the second stage of thermal analysis. Both water and amine group concentrations decrease with the decrease in chitosan molecular weight, resulting in reduced chitosan stability. As the thermal degradation progresses, chitin loses weight [34] Chitin and chitosan residual weight loss occurs at 500 ºC and beyond. Structural changes occurring during the depolymerization of chitosan are confirmed with this analysis. As the molecular weight decreases, the thermal stability of chitosan also decreases.

The DSC for standard and prepared chitin and chitosan was investigated by TGA-DTA in presence of nitrogen atmosphere. The DSC curves of the chitin and purified chitosan are

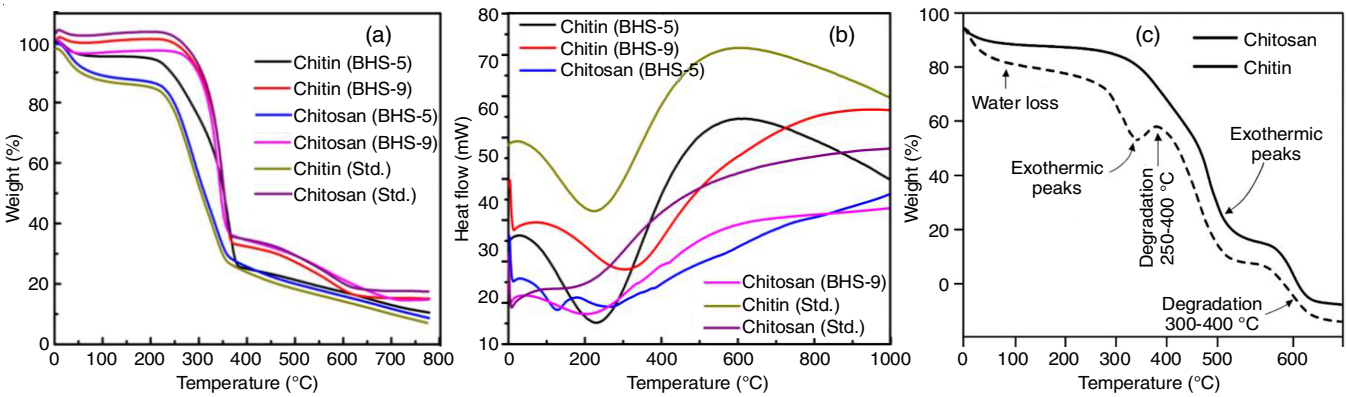

Fig. 5. (a) TG curves of chitin and chitosan obtained from *Aspergillus niger* in comparison with standard (Hi-Media), (b) DSC plots of chitin and chitosan isolated from *Aspergillus niger* in comparison with standard (Hi-Media) and (c) relative scheme of DSC analysis for chitosan and chitin

presented in Fig. 5b. In all cases, two exothermic peaks were identified in DSC at different temperatures. The first peak corresponds to physically adsorbed water in 130-180 ºC temperature range [34]. The second peak showed quick weight loss at 300-600 ºC, getting a highest at 585 ºC for chitin and 600 ºC for chitosan, respectively. The second period of chitin degradation occurred at a slight low temperature than the equivalent period of chitosan, indicated that chitin is less stable than purified chitosan owing to the weakening of intermolecular and extramolecular hydrogen bondings [41]. Thus, the TG analysis supports the DSC results in this study (Fig. 5c).

Thermal degradation stages are explained with the simplified steps like:

Moisture loss (60-120 ºC):

$$\frac{\text{Chitin}}{\text{Chitosan}_{(s)}} \longrightarrow \frac{\text{Chitin}}{\text{Chitosan}_{(dry)}} + H_2O \quad (1)$$

Deacetylation and main chain (250-400 ºC):

$$\frac{\text{Chitin}}{\text{Chitosan}_{(dry)}} \longrightarrow \text{Volatile gases} + \text{Residue}_{carbon} \quad (2)$$

Carbon residue oxidation (under oxidative conditions: 400-600 ºC):

$$\text{Residue}_{carbon} + O_2 \longrightarrow CO_2 + H_2O \quad (3)$$

Antioxidant activity of fungal chitosan: The antioxidant activity of commercial chitosan and chitosan isolated from *A. niger* (BSH5) and *A. niger* (BSH9) were analyzed and the results are depicted in Fig. 6. According to the result, it is revealed that the antioxidant activity of standard chitosan is lower than that of fungal chitosan. This may be due to the presence of more free amine groups in fungal chitosan than in crustacean chitosan. It is also observed that as the concentration of chitosan increases, the antioxidant activity also increases. The maximum antioxidant activity of chitosan was studied for a concentration of 2 mg/mL of chitosan.

Thus, it can be concluded that fungal chitosan, since they are low in molecular weight and high in DD% than the standard chitin and chitosan, with higher MW and lower DD%, exhibits higher antioxidant activity. Among the four fungal chitosan, chitosan from *A. niger* (BSH9) showed the maximum radical scavenging activity of 47.65%, followed by 46.38% from *A. niger* (BSH5). The free radical scavenging activity of all fungal chitosan was much higher than the standard chitin and chitosan. Therefore, sustainable production of fungal chitosan with efficient antioxidant activity has been achieved.

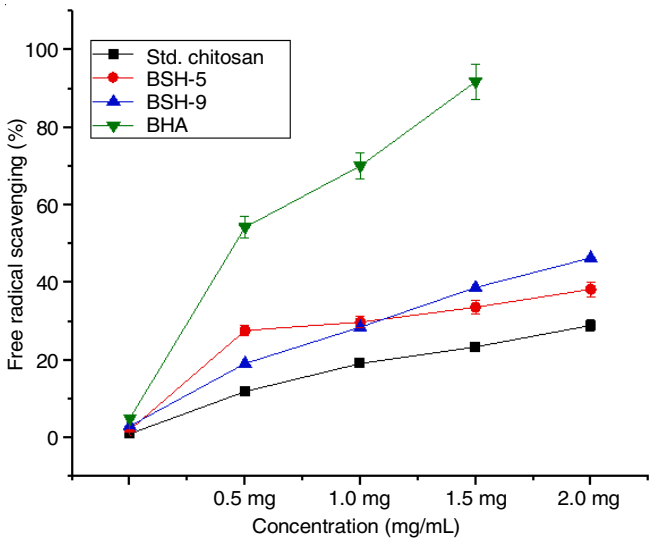

Fig. 6. Antioxidant activity chitosan from *Aspergillus niger* (BSH5 & BSH9) compared to standard chitosan and BHA

Cytotoxic effects of fungal chitosan: The cytotoxic effect of standard chitosan and chitosan from *A.niger* (BSH5 and BSH9) was obtained against breast (MCF7), lung (A549) and leukaemia (K562) cancer cell lines and was assessed and compared to the standard drug paclitaxel. The IC_{50} values for all the chitosan samples against the three studied cell lines are depicted in Table-2. The IC_{50} values indicates that, the cytotoxic effect of chitosan from *A. niger* (BSH5 and BSH9) are less than the standard paclitaxel for breast (MCF7) cancer cell lines. This signifies that the fungal chitosan has potent cytotoxicity against MCF7 cell lines than the control drug and standard chitosan from crustacean source. However, all the three chitosan samples did not show reasonable cytotoxicity against lung (A549) and leukaemia (K562) cell lines. However, the IC_{50} values of fungal chitosan against all the three cancer cell lines are much lesser than the standard chitosan (Table-2). This signifies that fungal chitosan are potent anticancer agents than the standard chitosan from crustacean source, which may be attributed to the molecular characteristics of fungal chitosan such as molecular weight (MW), degree of deacetylation (DD%) and viscosity (cP). Since fungal chitosan exhibited less MW and high DD% than the standard chitosan. Earlier studies proved that due to high DD% and low MW fungal chitosan exhibits higher cytotoxic effect than the crustacean chitosan [42].

TABLE-2
CYTOTOXICITY-IC_{50} OF STANDARD CHITOSAN AND CHITOSAN FROM *A. niger* (BSH5 AND BSH9)

Material	IC_{50} (µg/mL)		
	MCF7	K562	A549
Paclitaxel	298.86	247.63	273.25
Std. chitosan	336.00	494.50	709.20
Chitosan BSH5	206.60	459.50	350.50
Chitosan BSH9	244.70	434.60	517.50

All the three cancer cell lines were considered to determine the overall cell viability. The study aims to identify the potent fungal species among the isolated fungi from which the chitosan has been extracted. According to the result, it can be concluded that the MCF7 cell line showed (Fig. 7a) the highest sensitivity (16.37%) to fungal chitosan at 800 µg/mL, followed by K562 (32.12%) (Fig. 7b) and A549 (35.82%) (Fig. 7c). It is obvious from the results that chitosan prepared from *A. niger* (BSH9) has shown maximum cytotoxic effect against all the three cell lines in comparison with chitosan from *A. niger* (BSH5) and standard chitosan (Figs. 8-10).

Cytotoxic activity of chitosan in different cancer cell lines has been found to be due to apoptosis [43], which is initiated by activation of procaspase triggered from outside the cell to accelerate the cleavage of cascade to amplify the death signals

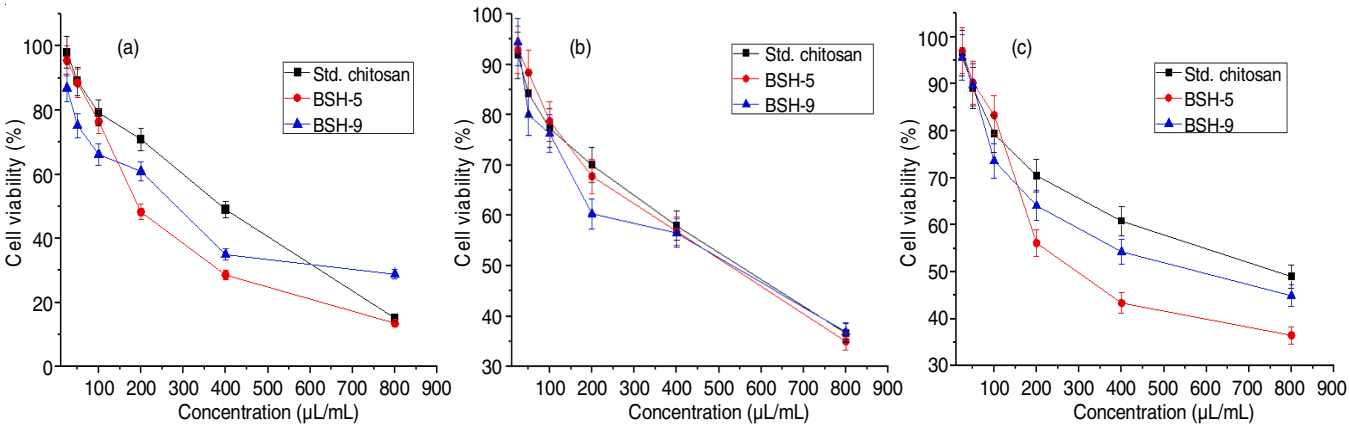

Fig. 7. Cytotoxic activity of standard chitosan, chitosan from *A. niger* (BSH5 and BSH9) against (a) breast cancer (MCF7) cell lines, (b) leukaemia cancer (K562) cell lines and (c) leukaemia cancer (A549) cell lines

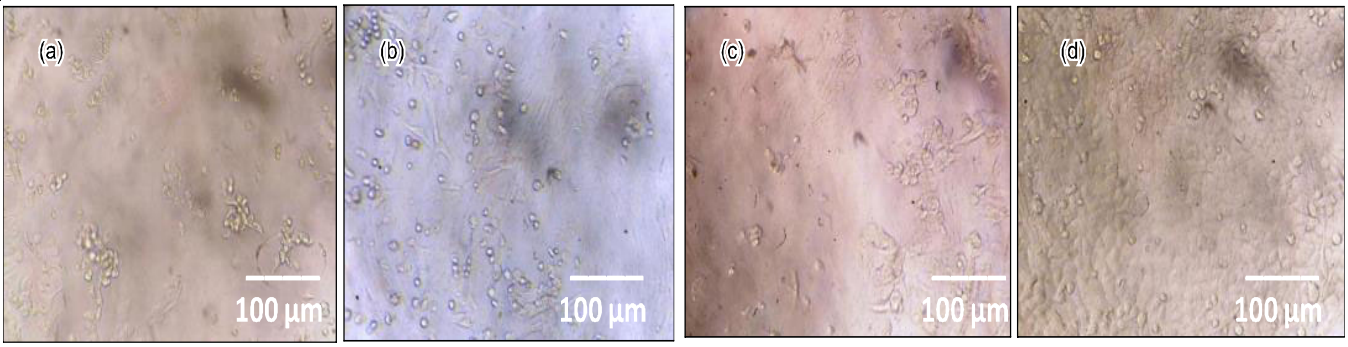

Fig. 8. Photomicrographs showing cytotoxic effect of standard chitosan (a), chitosan from *A. niger* (BSH5) (b), chitosan from *A. niger* (BSH9) (c) and negative control (d) against MCF7 cell lines

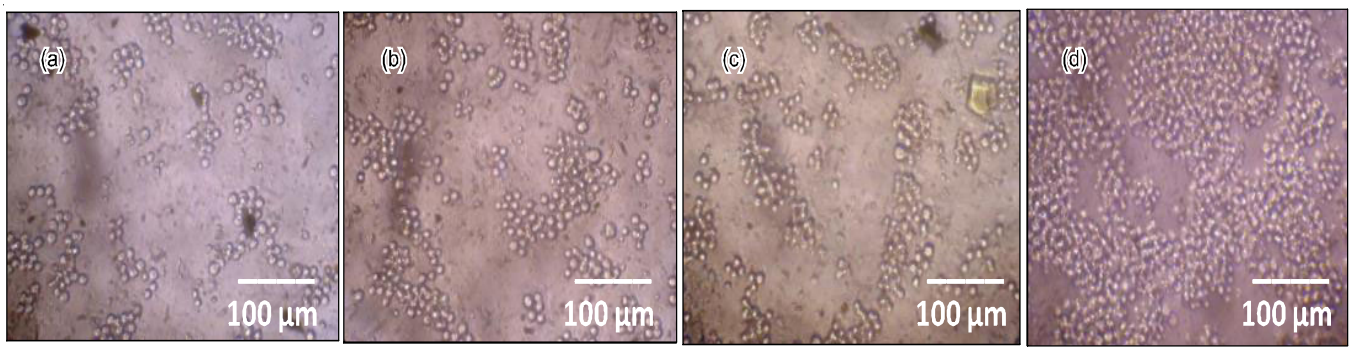

Fig. 9. Photomicrographs showing cytotoxic effect of standard chitosan (a), chitosan from *A. niger* (BSH5) (b), chitosan from *A. niger* (BSH9) (c) and negative control (d) against K562 cell lines

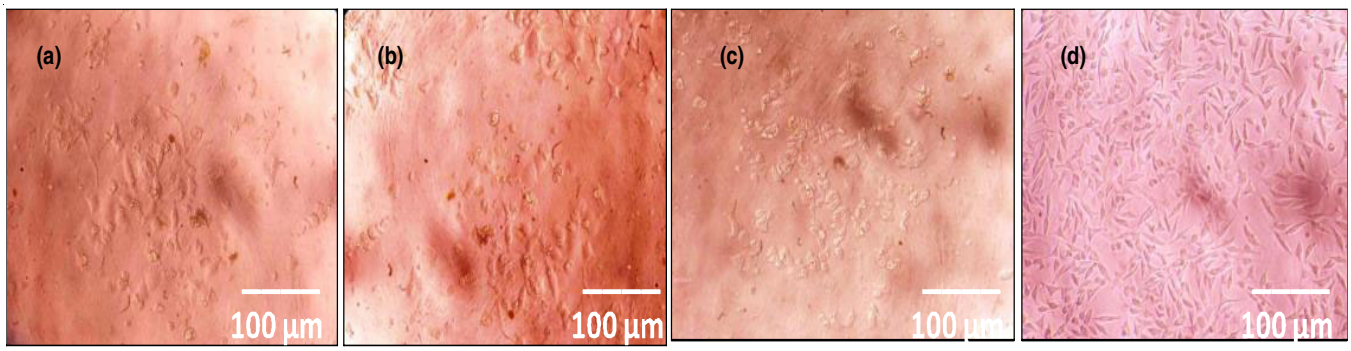

Fig. 10. Photomicrographs showing cytotoxic effect of standard chitosan (a), chitosan from *A. niger* (BSH5) (b), chitosan from *A. niger* (BSH9) (c) and negative control (d) against A549 cell lines

[44]. These studies helped to identify the possible anticancer mechanism of fungal chitosan and therefore, the possible mechanism would be permeation enhancing in case of breast cancer cell lines (MCF7) and cellular apoptosis mechanism in case of lung (A549) and leukaemia (K562) cancer cell lines respectively, because of increase in chitosan concentration the cell migration is inhibited by lowering the toxicity results in the elevation of anti-metastatic behaviour of the biopolymer [45].

Conclusion

Fungal strains *Aspergillus niger* were isolated from two different sources and further used for green synthesis of biopolymers. Chitin and chitosan yields from fungal strains were recorded in terms of dry weight (w/w). *A. niger* (BSH9) strain has shown the maximum yield of chitin and chitosan as compared to *A. niger* (BSH5). The characterization of chitin and chitosan was performed through various analytical tools such as FTIR, XRD, NMR, TGA, DSC and SEM to identify the functional groups in the extracted biopolymers and confirm the biopolymer production. The biological activities of chitosan were extensively studied for their antioxidant and cytotoxic activities. From the current study, it is obvious that due to the low molecular weight and lower viscosity, fungal chitosan was able to show the higher antioxidant activity than crustacean chitosan. Whereas, the cytotoxic activity was studied using three different cancer cell-lines and the results specify that fungal chitosan has shown comparative cytotoxic activity to commercial chitosan. From these studies, it can be concluded that the degree of deacetylation and molecular weight of the biopolymer play a crucial role in the biological applications of chitosan. The IC_{50} values of fungal chitosan suggest that chitosan biopolymers are potent cytotoxic agents against breast cancer (MCF7) cell lines, as indicated by their IC_{50} values. This could be a novel approach where fungal chitosan in its purest form can be used alone as an anticancer agent against many cancer types.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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