

## Extraction of Chitin and Chitosan from Wild Type *Pleurotus* Spp and its Potential Application - Innovative Approach

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**Biopolymer of chitin and chitosan were extracted from the two *Pleurotus* spp. of *P.floridanus* and *P.djamor*. After the optimization, genomic DNA was isolated, and by using ITS 1 and ITS 4 primers molecular identification was carried out and the nucleotides were submitted to NCBI. The biopolymer have a great commercial importance now a days because of it high efficiency in all the field. The present study was focused on to extract the chitin and chitosan from the *Pleurotus* spp. and these polymer was subjected to analysis the total antioxidant activity, antimicrobial activity against food borne pathogens of *E.coli*, *B. cereus*, *S.aureus*, *A.niger* and *A.flavus*. Cytotoxicity study against HeLa Cell Line was also performed to study the efficacy of the extracted polymer.**

**Keywords:** *Pleurotus* spp, chitin, chitosan, total antioxidant, antimicrobial activity, Cytotoxicity.

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Chitin is similar to cellulose with the chemical structure of [poly- $\beta$ -(1  $\rightarrow$  4) – N-acetyl-D-glucosamine], chitosan is one hydroxyl group on each monomer has substituted with an acetyl amine group.<sup>1</sup> These polysaccharide is chiefly present in the exoskeleton of crustaceans (shells of crab, shrimp, prawn, etc) and contain a percentage of inorganic material CaCO<sub>3</sub>, while production which is releasing CO<sub>2</sub> to the environment. The present pertain about the global warming which is not an advisable or environmental friendly process.<sup>2</sup>

Several method was already done to extract the chitin and chitosan from the crustaceans, despite these have thousands of applications and use in the field of medical and industrially. The importance and the needs of these polymers also facing the problem of seasonal variations and it is

non-vegetarian source. To put back these problems scientist find an alternative source to extract these polymer from some plants and fungi or from mushroom.

Chitin is widely distributed in fungi where it is component of the cell wall and structural membrane of mycelia, stalks, and spores. Chitin in fungi possess principally the same structure of chitin occurring in other organism.<sup>3</sup> However that all fungi contain chitin and the polymer may be absent in one species that is closely related to the other variation. In the amount of chitin may depend on physiological parameters, environmental and fermented conditions, processing and in the culturing of the fungi.

The importance and use of chitin and chitosan is increasing day by day is because of its antioxidant activity,<sup>4</sup> biocompatibility of which does not have an toxic or injurious effects on biological system,<sup>5</sup> biodegradability – which is being able to broken down by natural process

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that is by bacteria, fungi, and other simple microorganism,<sup>6</sup> antimicrobial activity,<sup>7</sup> cosmetics, medicinal applications, food, flavour or micro encapsulater, flocculants for liquid wastes,<sup>8</sup> enzyme immobilizer, production of contact lens and production of eye bandage.<sup>9</sup>

The advantage or benefits of using fungi is the easy handling, harvesting and controlling to produce high quality chitin and chitosan. Guha et al<sup>10</sup> reported that the different type of fermentation technologies are helping to replace these problems by concerning the conventional method to produce these polymers from fungi.

The present study was aimed on to extract the chitin and chitosan from the *Pleurotus* spp. The Molecular identification of *Pleurotus* spp. was done followed by the extraction of chitin and chitosan. After characterisation total antioxidant activity, antimicrobial activity against food borne pathogens and cytotoxicity study against HeLa Cell Line were performed.

## MATERIALS AND METHODS

### Source of inoculums

The two *Pleurotus* spp. Strain were collected from Tamilnadu agricultural university, Coimbatore, Tamilnadu, India and brought to the laboratory in an air tight container. The strain was subculture to PDA plate (39gm in 1000ml, Himedia, Mumbai, India) and incubated at 30°C for 10-12 days. After mycelial growth mycelia was separated and used for further studies.

### Molecular identification

#### DNA isolation

DNA isolation was done by the modified protocol of Kumar et al<sup>11</sup> the growed mycelia was crushed with CTAB buffer and incubated at 65°C for 45 minutes. After incubation the supernatant was collected by centrifugation. Chloroform and isoamyl alcohol was added in the ratio of 24:1 and allowed for centrifugation to separate the aqueous layer. To the collected aqueous layer 2 volume of isopropanol was added and pellet was collected by centrifugation. Pellet was washed with absolute ethanol and 70% ethanol to remove the impurities. After air drying the pellet was dissolved by adding 1X TE buffer and the isolated DNA was confirmed by 0.8% agarose gel.

### PCR and Sequencing

The isolated DNA was used for molecular identification using AB Applied Biosystem veriti 96well Thermal cycle PCR detection system. For the amplification, universal fungal primer of internal transcribed spacer 1 (ITS1:5'-TCCGTAGGTGAACCTGCGG-3') and internal transcribed spacer 4 (ITS4:5'-TCCTCCGCTTATTGATATGC-3') were used. To the 2µl of isolated DNA 1µl of each ITS primers, 10µl of the master mix (Sigma), and 8µl of distilled water was added to prepare the PCR reaction mixture. PCR was done, initial denaturation at 95°C for 2minutes, denaturation 95°C for 1minutes, annealing at 55°C for 1 minutes, and extension at 72°C for 1minutes and final extension for 5 minutes at 72°C the total number of cycle was 30. The amplified product was analysed by 1.5% agarose gel. The PCR product was purified using Quiagen kit as per the manufacture instruction and sent to Chromous Biotech Pvt. Ltd Bangalore for sequencing. The sequence result was analysed by BLAST, and submitted to NCBI for accession number and the phylogenetic tree was constructed using MEGA-7 software.

### Production and extraction of chitin and chitosan in fermentation medium

The production of chitin and chitosan was done from the mycelia mat of both blended *Pleurotus* spp. which was grown in the MGYB (Meat extract-2%, Glucose -1.5%, Yeast -0.3%, Peptone -0.5%) medium. After 10-12 days of incubation described by Jesteena et al<sup>12</sup>. The mycelia mat was separated aseptically and the polymer was extracted from both the *Pleurotus* spp. by the treatment method of alkaline, acid and deproteinisation to get the chitin. Chitosan was extracted from the chitin by deacetylation to precipitate the polymer Sandra et al<sup>13</sup>. The final product was washed with ethanol and allowed for drying, after drying the sample was used for characterisation and further applications.

### Characterisation of extracted chitin and chitosan

#### UV-VIS study

The extracted chitin and chitosan sample was further confirmed by UV-Visible (Labtronics Microprocessor) study in the nanometer of 200-500nm, after setting the baseline with the scanning interval of 2nm.

**FTIR-Fourier Transform Infrared Spectroscopy**

To analyse the functional group which is present in the chitin and chitosan sample, the scan was done in the spectral region of 500<sup>cm</sup>-1 to 4000<sup>cm</sup>-1 with the resolution of 4cm-1 using Shimadzu FTIR instrument. The dried sample was prepared by KBr pellet and stabilized before undergoing the spectrum analysis.

**Total antioxidant activity**

Total antioxidant activity of the extracted sample was analysed by phospho molybdenum method described by Prieto *et al*<sup>14</sup> with slight modification. The 1ml of the extracted chitin and chitosan (0.1 g/ml) was mixed with equal amount of reaction mixture (0.6M H<sub>2</sub>SO<sub>4</sub>, 28mM sodium phosphate and 4mM ammonium molybdate). The addition of reaction mixture, sample was incubated at 45°C for 90minutes in water bath. After incubation the sample was allowed to cool in room temperature and the OD measurement was measured at 695nm using UV-Visible spectrophotometer (ELICO SL 159). Blank was prepared without the addition of sample and the mg/g of total antioxidant was calculated by using ascorbic acid as a standard.

**Antimicrobial activity**

The antimicrobial activity was done by well diffusion method against food borne pathogens of, *E.coli*, *S.aureus* and *B.cereus* for antibacterial and antifungal activity against *A.niger*, and *A.flavus*, by the previous method of Jesteena *et al*.<sup>12</sup> Cefotaxime (CTX 30) disc was used as a positive control for bacteria, fluconazole (30mg/ml) was used for fungi. After specific incubation period the zone of inhibition was measured.

**Cytotoxicity study**

Cytotoxicity study was carried out using MTT(3-(4,5-Dimethylthiazol-2-yl)-2,5diphenyletrazolium bromide) assay method by Grushree *et al*.<sup>15</sup> HeLa Cell Line in DMEM medium was allowed to grow in the CO<sub>2</sub> incubator by 5% CO<sub>2</sub>, 80% humidity and at 37°C. After 48hrs of incubation the live cells were confirmed by inverted microscope (Unicon) and the cells were transferred to microplate reader. The sample in the different concentration of 10,20,30,40,50µl was taken along with 100µl of the Cell Line, control (only Cell Line) also added and incubated in the CO<sub>2</sub> incubator for 24 hrs. After incubation the cells were washed with DMSO and trypsin.

20µl of MTT dye was added and using ELISA (Robonik-Readwell Touch Elisa Plate Analyser) reader at 570nm the OD was taken after 24hrs. The percentage of cell death was calculated using the following formula;

$$\% \text{ of cell death} = \frac{[\text{Control OD} - \text{Sample OD}]}{\text{Control OD}} \times 100$$

**RESULTS AND DISCUSSION**

The optimum day was confirmed by the report of other research article and also the previous work done Jesteena *et al*.<sup>12</sup> Sandra *et al*<sup>13</sup> reported that the maximum biomass production was obtained after 8<sup>th</sup> day of incubation 21.87±2.2g/L<sup>-1</sup>. The same was incubated upto 14<sup>th</sup> days but the substrate decreased during the day of culture until a minimum of 5.57±0.85g/L<sup>-1</sup>. Maghsoodi *et al*<sup>16</sup> observed the maximum production of chitosan was in the day of 12<sup>th</sup> and the production was started at the day of 4<sup>th</sup>. In the present study after 10-12<sup>th</sup> day of incubation, it shown good mycelial growth and this was used for molecular identification and also the extraction of chitin and chitosan.

**Molecular identification**

The DNA of the both *Pleurotus* spp. was done separately and was documented on 0.8% agarose gel. The result was given in figure 1.

The molecular analysis of partial DNA gene sequencing shows 88 % of similarities with *P.floridanus* and 83% similarities for *P.djamor* and the NCBI accession numbers were MG324371.1 and MG328900.1

Sasidhara and Thirunalasundari<sup>17</sup> done molecular identification of the mushroom using genomic DNA with the approximate molecular weight of 559 base pairs with ITS1 and ITS4 primers and submitted to the gene bank in the name of *Agaricomycetes* Spp. II India 01, these wild mushroom shows the homology score of 88 with *Perenniporia* Spp. Alvarado *et al*<sup>18</sup> done the DNA extraction from the mycelium to get higher quantity of the genetic material and they used ITS1 and ITS2 for their PCR cycling.

Menolli *et al*<sup>19</sup> reported the molecular identification of *P. ostreatus* with 90% support with other sequence from the NCBI gene bank *P.ostreatus* and *P.pulmonarius*. Chandra *et al*<sup>20</sup> constitute that the similarities of 27% between

*P.floridanus* and *P.sajorcaju*. This is may be because of different cultivated area or based upon the growth condition.

**Characterisation**

**UV-Visible analysis**

UV-Visible spectroscopy analysis is helping to identify the type of absorption from the ground stage to excited stage. In the present study the polymer was scanned at 200-500nm was used to analysis the presence of extracted chitin and chitosan from the *Pleurotus* spp. The study was showing three Plasmon peak for chitin in the nm of 265.0nm, 260.0nm and 215.0nm (Fig 5).For chitosan 285.0nm, 280.0nm and 245.0nm(Fig 6).

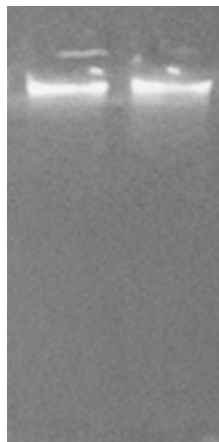
The earlier report by Krishnaveni and Ragnathan<sup>21</sup> observed the Plasmon peak at 390nm for their synthesised nanocomposites from the chitin chitosan. Negrea et al<sup>22</sup> got minimum

absorption of 212.6nm for chitin, and for Silva et al<sup>23</sup> got peak on 202nm.

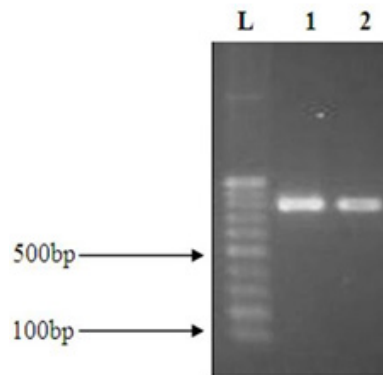
**FTIR**

In the analytical part infrared spectrum field was 4000<sup>cm-1</sup>-500<sup>cm-1</sup> and the resulting band was larger macromolecular character because of the inter molecular binding of hydrogen. Tasar et al<sup>24</sup> in their structural group identification FTIR was carried out from 4000<sup>cm-1</sup> – 550<sup>cm-1</sup> and they are finalised for industrial purpose with large production of high quality chitosan from low cost method.

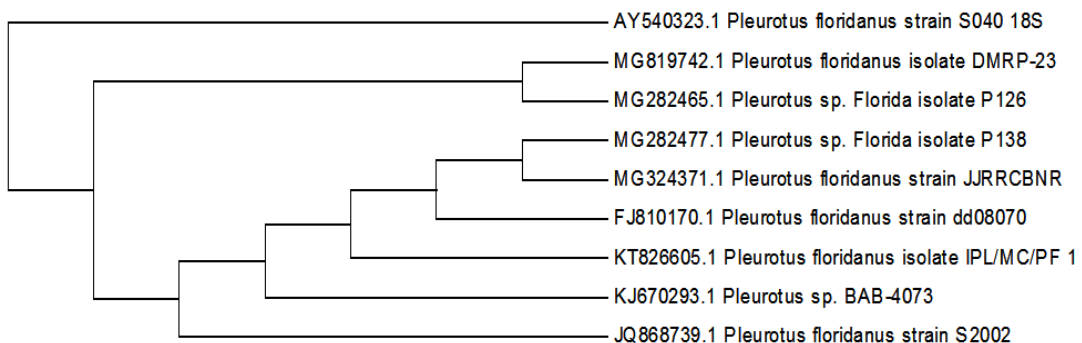
FTIR result of chitin (Fig.7) showed the presence amide III in 952.84<sup>cm-1</sup> to 1261.45<sup>cm-1</sup>amide III acetyl group. From the result of Prabhu and Natarajan<sup>25</sup> the band of 1261.45 is an acetyl ester band, 1570.06<sup>cm-1</sup> is an N-acetyl ester band and usually an high amide III absorption. 1631.78<sup>cm-1</sup>



**Fig. 1.** DNA of *P.floridanus* (Lane 1) and *P.djamor* (Lane2)



**Fig. 2.** PCR result of the two whit rot fungi Lane 1:100Kbs Ladder. Lane 2: *P.floridanus* Lane 3: *P.djamor*



**Fig. 3.** Phylogenetic tree of *P.floridanus*

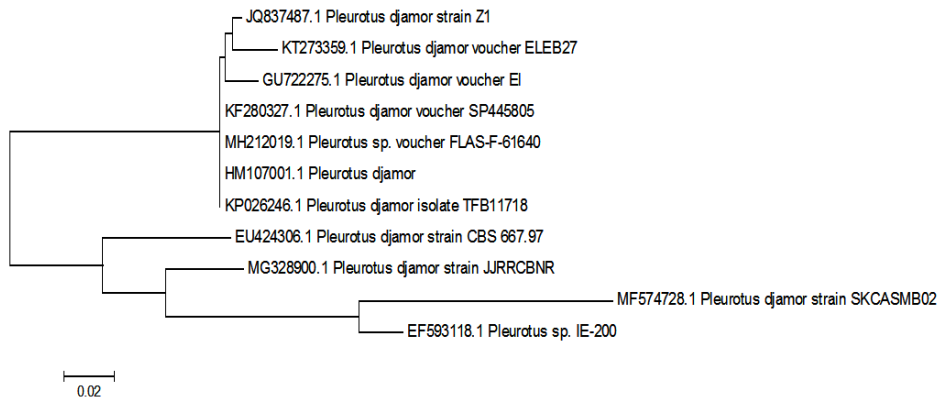


Fig. 4. Phylogenetic tree of *P. djamor*

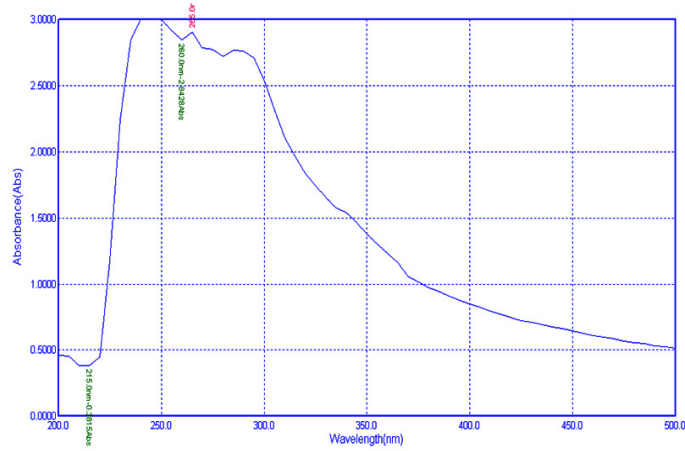


Fig. 5. UV-Visible study of chitin

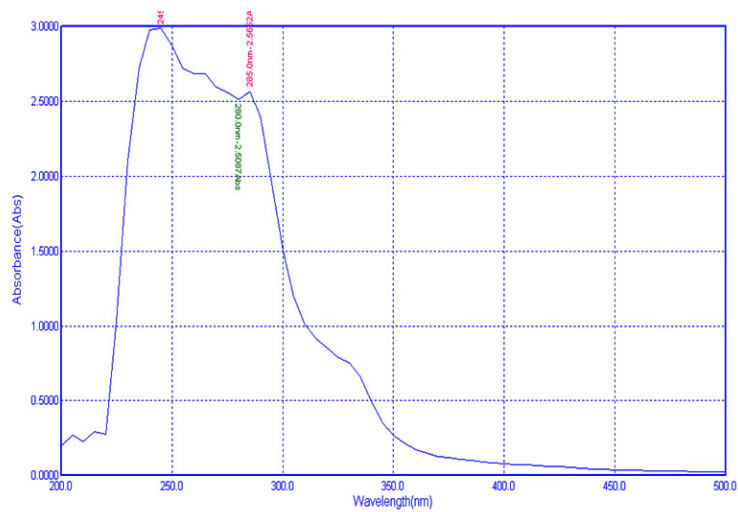


Fig. 6. UV-Visible study of chitosan

to  $1658.78\text{cm}^{-1}$  is an amide C=O stretch,  $1774.51$  is an ketone C=O stretch absorption is an strongest IR absorption and these are use full in the structure determination of chitin.  $3102.01\text{cm}^{-1}$  is an amino peak alpha chitin and which is also an stretching of alkene C-H which correspond to the vibrational stretching of hydroxyl group of our study.

Fig.8 shows the chitosan sample showed bands at  $3761.91\text{cm}^{-1}$  due to the stretching vibration of OH group  $2897.08\text{cm}^{-1}$  is the vibration of CH bond (aldehyde) Silva *et al.*<sup>26</sup> Stretching at  $1658.78\text{cm}^{-1}$  is the vibration of alkene group, the stretching from  $1072.42\text{cm}^{-1}$  to  $1026.13\text{cm}^{-1}$  has been assigned to vibration of CO group Xu

et al.<sup>27</sup> The band located at  $1153.43\text{cm}^{-1}$  (C-O-C vibration) is an vibration of CO in the oxygen bride is the outcome of deacetylated chitosan Silva et al.<sup>26</sup> The peak at  $894.97$  showing the presence of polysaccharide structure of chitosan and the band from  $1072\text{cm}^{-1}$  showing the binding of C-O-H, C-O-C,  $\text{CH}_2\text{CO}$  Paluszkiwicz.<sup>28</sup> Other band which is formed before  $894.97$  is showing the strong bending of C-H group, and in the particular study bands of  $1415.75\text{cm}^{-1}$  was the vibration of O-H group (alcohol),  $2897.08$  is the vibration of C-H stretching (aldehyde) and  $2372.72\text{cm}^{-1}$  is a strong O=C=O stretching.

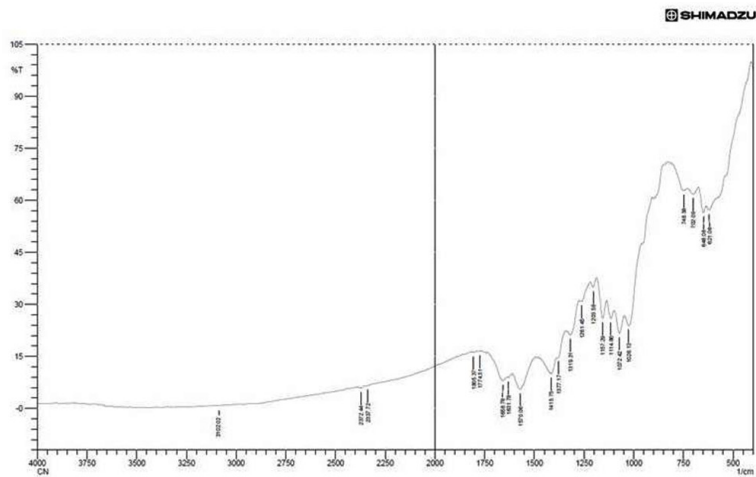


Fig. 7. FTIR analysis of chitin

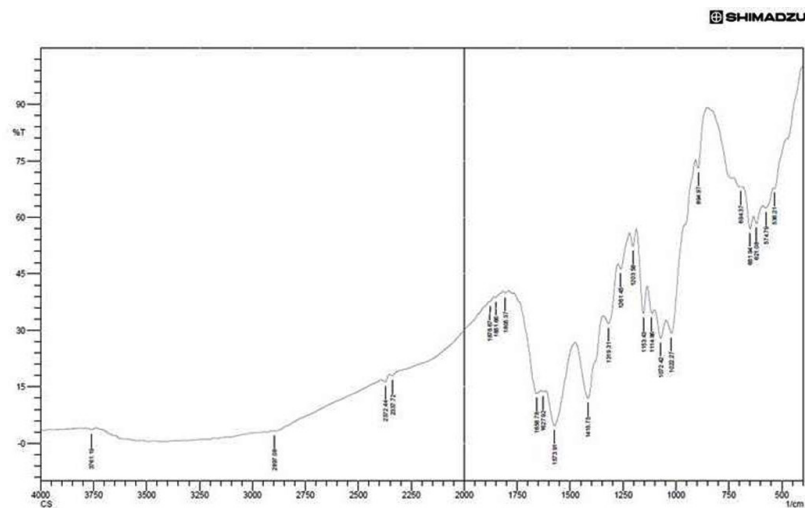


Fig. 8. FTIR analysis of chitosan

**Total antioxidant activity**

Using phospho molybdenum method the total antioxidant activity of the sample was quantified for chitin and chitosan. Total antioxidant activity of the sample of chitin 101mg/g and for chitosan 138mg/g. According to Prieto et al<sup>14</sup> the absorbance at 695nm showing phospho molybdenum complex reaction from MO (V1)

reduction to MO (V) which is turned to blue green colour.

Xie et al<sup>29</sup> reported that the antioxidant activity of the chitosan may be due to the reaction of residual free or free radical or the amino group to form constant macromolecule radicals or the group can form amino group by absorbing H<sub>2</sub> ions from the mixture and then reacting with radical through can additional reaction.

**Antimicrobial activity**

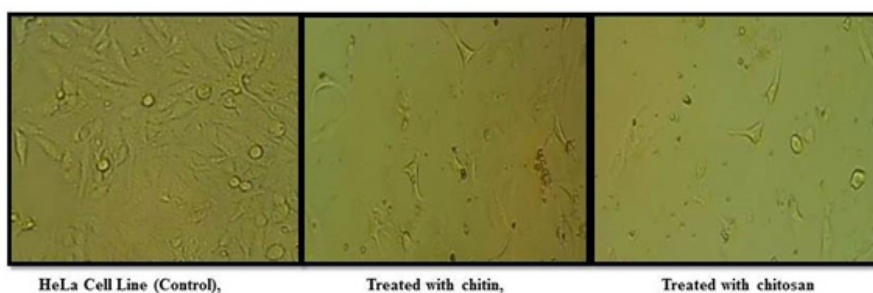
Antibacterial activity of the two extracted polymer was showing good result against the food borne pathogens. For all the selected microorganisms, chitosan showing good result



**Fig. 9.** Antibacterial activity against *B.cereus*, *E.coli* and *S.aureus* from the figure cn-chitin, cs-chitosan, D- antibiotic disc



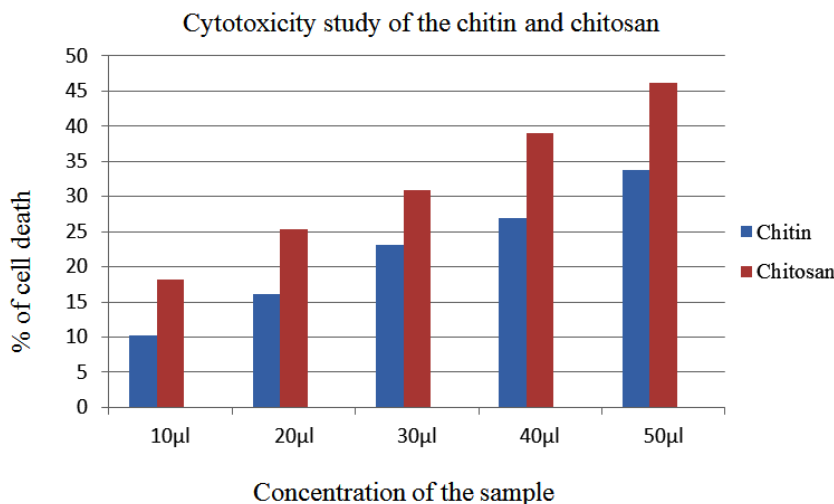
**Fig. 10.** Antifungal activity against *A.niger* and *A.flavus* from the figure,cn-Chitin, cs-Chitosan, Ab- Antibiotic



**Fig. 11.** Control Cell Line and the Cell Line treated with chitin and chitosan sample - microscopic view

**Table 1.** Zone of inhibition in mm for antimicrobial activity of the extracted sample

Sample used	Food pathogens used				
	<i>B.cereus</i>	Bacteria <i>E.coli</i>	<i>S.aureus</i>	Fungi <i>A.niger</i> <i>A.flavus</i>	
Chitin (cn)	2 mm	7 mm	3 mm	3 mm	2 mm
Chitosan (cs)	5 mm	10 mm	6 mm	3 mm	1 mm
Antibiotic	1 mm	12 mm	2 mm	4 mm	3 mm



**Fig. 12.** Cytotoxicity study of the extracted polymer

compared with chitin. The results were given in the Figure 9 and 10, Table 1.

Sudharshan et al<sup>30</sup> investigated the antimicrobial activity of the various water soluble chitosan on dissimilar gram negative and gram positive bacteria and also against fungi which shown good result in a very short time. Research of Shaidi et al<sup>31</sup> reported that this changes is because of the electro negatively charged site of bacterial surface causing some agglutination in the chitosan treated cells, were the interaction between the bacteria (especially *E.coli* and *Salmonella* Spp.) it is showing non- liberation of lipopolysaccharide in other lipid from the membrane analysing with electronmicroscopy showing which will cause exterior modification in the bacterial cells.

Nadarajah and Cheng<sup>32</sup> reported that the chitosan have a good antibacterial activity against gram positive and gram negative bacteria. In their investigation 2-10mg/ml was used and 8mg/ml shows higher percentage of inhibition against gram negative bacteria of *B.cereus*, *P.aeruginosa* and *Enterococcus aerogenes*. The previous results of the antimicrobial activity of the chitin and chitosan extracted from the *Pleurotus* spp. Jesteena et al<sup>12</sup> also showed better result against *E.coli*, *S.aureus* and *B.subtilis*. Antifungal study showed better result against *A.niger*, *A.flavus* and *F.solani*.

According to Ghaouth et al<sup>7</sup> chitosan have the ability to conquer to inhibit the growth of fungi.

It delayed the acceleration of the decay of stored fruits. Furthermore chitosan coated fruits inhibit the growth of *P. digitatum*, *B. lecanidion*, *B. cinerea* and *P.italicum* in citrus fruits Chien et al.<sup>33</sup>

#### **Cytotoxicity study**

This research was done to identify the cytotoxicity in the aim of finding an alternative source to kill the cancer cells. The percentage of cell death was increasing with increasing concentration for both the sample. For chitin 10.25, 16.15, 23.15, 26.89, 33.75 % of cell death, and for chitosan 18.16, 25.58, 30.96, 39.02, 46.19% of cell death from the concentration of 10 µl to 50µl of the sample was occurred. Compared to chitin, chitosan showing better results against the Cell Line. Fig. 11 denoting microscopic view of the control Cell Line, cell death by the addition of 50µl of chitin and 50 µl of chitosan sample.

From the review of Yoshinori et al<sup>34</sup> chitin and chitosan derivatives are campaigner to carry anticancer agent. The conjugated chitosan demonstrating good result in the life span of P388 leukemia bearing mice after the intraperitoneal injection. On the other hand the result of Leena et al<sup>35</sup> stated that the chitosan concentration from 1000 µg/ml to 7.8µg/ml and they got 50% of cell death against A549 lung cancer Cell Line using MTT assay method.



## CONCLUSION

The present research can be resolved effectively to produce the chitin and chitosan from the *Pleurotus* spp. Culturing in MGYM medium, the optimization was confirmed by using the formation of mycelia mat on the media. The two *Pleurotus* spp was subjected to molecular level identification to confirm the fungi and got 88% similarities to *P.floridanus* and other fungi shown 83% similarities to *P.djamor*. The mycelia mat after 12<sup>th</sup> day of incubation blended and used to extract the chitin and chitosan. The extracted chitin and chitosan were characterised by UV-Visible study and FTIR to finalise the functional group. So far limited paper only reviewed about the antioxidant activity and this work is unique to quantifying the antioxidant activity and anticancer study. Total antioxidant activity using phosphor molybdenum method for chitin 101mg/g and for chitosan 138mg/g. Antimicrobial activity against the food borne pathogens of *E.coli*, *B.cereus*, *S.aureus*, *A.niger* and *A.flavus* displaying better result, cytotoxicity study also showing 10.25 to 46. 19 % of cell death against the HeLa Cell Line. Discussing with these applications, chitosan showing dependable results comparing with chitin for total antioxidant activity, antimicrobial activity and cytotoxicity study.

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