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Extraction Purification and Applications of Chitin from *Isoptera Spp* and Silkworm Cocoon

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ABSTRACT:-*Chitin is a large, structural polysaccharide made from chains of modified glucose. They are attracting great interest due to their beneficial biological properties, their potential applications in various industrial fields and their notable bioactivity in biomedical fields. Traditionally chitin was extracted from various crustaceans; this paper aims to extract and purify chitin from Isopteraspp and Silkworm Cocoon using suitable solvents and characterized by comparing with standard chitin FT-IR and XRD values. The extracted chitin exhibited characteristics similar to the standard and was tested for its industrial and therapeutic applications. The characterization studies proved that the silkworm cocoon had abundance of chitin in them.*

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I. Introduction

Chitin is the most widespread amino polysaccharide in nature and is estimated annually to be produced almost as much as cellulose. It is a cationic aminopolysaccharide, composed of poly β (1-4) linked N-acetyl-D-glucosamine (NAG) residues. It is described as colourless, crystalline or amorphous powder, which is insoluble in water, organic solvents, dilute acids and alkali. Chitin is generally extracted from natural resources viz. terrestrial organisms, marine organisms, microorganisms like fungi and enzymatically from crustaceans shell waste materials. It is an important source of carbon and nitrogen for marine organisms. Chitosan, a linear polysaccharide is obtained by deacetylation of chitin. The percentage of chitin present varies depending on their sources.

Nowadays chitin is commercially manufactured from bio-wastes obtained from aquatic organisms. But due to the seasonal and capricious availability of raw materials, terrestrial crustaceans and mushrooms are the alternative source for their production. This biopolymer is biodegradable in nature and possesses many beneficial properties such as biocompatibility, antimicrobial, haemostatic, anti-inflammatory, anti-oxidant, muco-adhesion, analgesic, non-toxicity, adsorption enhancing, anti-hypersensitivity, anti-cholesterolemic, anti-cancer and anti-diabetic. It can also be used as fertilizer, food additive and emulsifying agent. Due to havingsuch respectable properties chitin and their derivatives are greatly exploited by the scientists to obtain tremendously better results in medical and engineering fields.

Chitin $(C_8H_{13}O_5N)_n$ is a homopolymer consisting of N-acetylglucosamine residues linked by β-(1-4)-glycosidic bonds. Chitin is made up of modified glucose monosaccharides. Glucose exists as a ring of carbon and oxygen molecules. Bonds between glucose molecules are known as glycosidic bonds. The oxygen that typically form hydroxyl groups bonded to the carbon ring can also form a bond with another carbon instead of hydrogen. In this way, monosaccharides can be linked together in long chains. Chitin is formed by a series of glycosidic bonds between substituted glucose molecules. In the linear chitin chain, every single sugar is rotated by 180° with respect to its neighbouring sugars. Thus, the repeating unit in the chitin chain is chitobiose.

CHARACTERISTICS OF CHITIN

- Chitin is stable in concentrated alkaline solutions, even at high temperatures.
- Chitin has no antigenic properties, so it is compatible with both plant and animal tissues.
- Chitinases are the hydrolytic enzymes which can decompose chitin. It breaks down the glycosidic bonds in chitin.
- Chitin can be easily processed into gels, beads, powders, fibers, membranes, cotton, flakes, sponges, colloids, films and spins.
- Chitin is colourless to off-white in colour, hard, inelastic, nitrogenous polysaccharide.
- Chitin's molecular weight ranges from 1.03×10^6 to 2.5×10^6 Da on average.

- Chitin has amino and hydroxyl groups that are easily substituted with other groups.

SOURCE OF CHITIN

From commercial point of view, terrestrial organisms are mainly used for the extraction of chitin, due to their easy availability and processability. Terrestrial organisms generally include crustaceans, arthropods, nematodes, insects, silkworms, mosquitoes, honeybees and many more. As the composition of these organisms is quite different, there is a variation in the contribution to the percentage of chitin produced.

CHITIN FROM WINGED TERMITES (*ISOPTERA*)

Insect chitin is a secretion of the epidermal, tracheal or midgut epithelial cells. Chitinous structures are mainly of ectodermal in multicellular organisms and form the characteristic exoskeleton of most of the invertebrates (insects) in contrast to its collagenous mesoderm. In the exoskeleton tissue, protein and chitin combine to form a protein-chitin matrix, which is then extensively calcified to yield hard shells. Chitin constitutes half of the total organic matter in the chitinous structure. Higher concentration about 85% is found in the insects, which synthesize chitin. There is no relation between the proportion of chitin and degree of hardness or flexibility of the structure. Chitin is associated with the other polysaccharides in fungal cell wall and proteins in other organisms.

This skeleton on the outside of the insect body appears hard and rigid due to the presence of chitin that is known for its tough elastic properties. Although chitin is the dominant constituent, other compounds such as proteins and calcium carbonate also play a crucial role in the formation of exoskeleton. The main function of this chitin-containing exoskeleton is to keep the inner soft tissue safe from any sort of injury. Most importantly, it prevents these delicate tissues from becoming dry. In short, it acts as a watertight barrier against dehydration, which is crucial for their survival. The hard chitin-containing exoskeleton also acts as a defense mechanism against predation. This outer covering can tolerate strong compressive stresses, which can provide protection from predation because predators exert a compressive force on the exoskeleton to injure their victim.

FIG 1 - WINGED TERMITES

Generally the inter-segmental membranes of the wings of most of the insects are flexible chitinous structure. Within the cuticle the chitin is assembled as nanofibres about 300nm in length and 3nm in diameter which provide the internal stability. Chitin synthesis complex is located in the cell membrane. Chitin is produced as microfibrils and arranges in the form of stacks leading to the formation of cuticle layer. In spite of extracting chitin from different insect, marine and microbial sources to study its characterization and various properties, the Winged Termites being considered as an insignificant insect by people lead to the attraction of using it as a source to extract chitin and discover its various activities in this paper

CHITIN FROM SILKWORM COCOON WASTES

Bombyx mori, the common silkworm is an economically important insect which is being the primary producer of silk. It feeds on the mulberry leaves. Silkworm undergoes complete metamorphosis i.e. their life cycle passes through four stages egg (embryo), larva, pupa and moth (adult).Silkworms are the only identified insects that provide food, fiber, and biomedical significance. Silkworm pupae are obtained after the extraction procedure of silk thread and are not used commercially as an edible insect in India except in the northeastern states. Later silkworm pupae protein has been considered to be a new available source of high-quality protein that contains all the amino acids needed by the human body. Silkworm pupa on its outer covering contains large

amount of chitin.Silkworm spins its own silken cocoon by extruding the silk fibres from its salivary glands. Since the silkworm is known to contain chitin in its exoskeleton and they spin their own cocoon this idea lead to extract chitin from its cocoon wastes which are thrown away after reeling the silk.

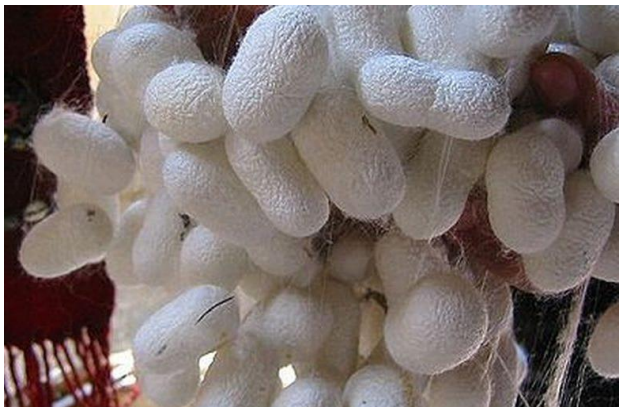

FIG 2 - SILKWORM COCOON

II. Materials And Method

SAMPLE COLLECTION

The Winged Termites were collected from Thekkadi after they have shed off overnight and sun dried for 5 days to remove the moisture content. The insects were then separated and ground to powdered form using a mortar and pestle. The Silkworm Cocoon was collected from the Tamil Nadu Sericulture Department of Salem and Krishnagiri District. The cocoons were then cut into small pieces and dried

SAMPLE PREPARATION

METHOD

The extraction of chitin is done by various chemical processes. The sample (insect and cocoon) was weighed and treated with 4% HCl for 24 hours known as demineralization. The residues were collected and washed with distilled water. The demineralized residues were suspended in 5% NaOH at room temperature for 48 hours known as deproteinization. The residues were collected and washed with distilled water.Thewas treated with 1% $KMnO_4$ for 30 minutes followed by 1% Oxalic acid for 2 hours for decolourising the sample and washed, air dried, powdered.

CONFIRMATORY TESTS

Solubility Test- 10 mg of insect and cocoon sample was taken separately and dissolved in 10ml of 70% orthophosphoric acid and hexafluroisopropanol respectively.

Thin Layer Chromatography- The insect and cocoon sample was spotted on a TLC plate and kept in a beaker containing iodine powder and closed using a lid. It was observed for colour change.

CHARACTERIZATION STUDIES

The sample was studied using the Fourier Transformation Infrared Spectroscopy (FT- IR) and X-Ray Diffraction (XRD) studies

FOURIER TRANSFORMATION INFRARED SPECTROSCOPY (FT-IR)

FTIR characterization of samples was performed with PerkinElmer Instrument. The spectra of chitin samples were obtained within a range of 450 – 4000 cm^{-1}.FTIR relies on the fact that the most molecules absorb light in the infra-red region of the electromagnetic spectrum. This absorption corresponds specifically to the bonds present in the molecule. The background emission spectrum of the IR source is first recorded, followed by the emission spectrum of the IR source with the sample in place. The ratio of the sample spectrum to the background spectrum is directly related to the sample's absorption spectrum. The resultant absorption spectrum from the bond natural vibration frequencies indicates the presence of various chemical bonds and functional groups present in the sample. FTIR is particularly useful for identification of organic molecular groups and compounds due to the range of functional groups, side chains and cross-links involved, all of which will have characteristic vibrational frequencies in the infra-red range.

POWDER X-RAY DIFFRACTION (PXRD)

X-ray powder diffraction (XRD) is a rapid analytical technique primarily used for phase identification of a crystalline material and can provide information on unit cell dimensions. The sample is analyzed within 2θ

(theta) range of 5-80. X-ray diffraction is now a common technique for the study of crystal structures and atomic spacing. X-ray diffraction is based on constructive interference of monochromatic X-rays and a crystalline sample. By scanning the sample through a range of 2θangles, all possible diffraction directions of the lattice should be attained due to the random orientation of the powdered material. Conversion of the diffraction peaks to d-spacings allows identification of the mineral because each mineral has a set of unique d-spacings. Typically, this is achieved by comparison of d-spacings with standard reference patterns.

APPLICATIONS

SAMPLE PREPARATION

The sample was prepared by dissolving the insect and cocoon sample in 70% orthophosphoric acid.

ANTIMICROBIAL ACTIVITY (AGAR WELL DIFFUSION METHOD)

The MHA media was prepared for antibacterial activity and SDA for antifungal activity. The different cultures of bacteria and fungi were swabbed on the agar plates using cotton swabs. The wells were bored and 50µl of 70% orthophosphoric acid was added to one well as control and 50µl of the prepared chitin samples were added on the other wells of each well. The plates were then incubated at 37˚C for 24 hours. The antimicrobial activity was assayed by measuring the diameter of the inhibition zone formed around each well.

ANTIOXIDANT ACTIVITY

HYDROGEN PEROXIDE RADICAL SCAVENGING ASSAY

The hydrogen peroxide solution was prepared using phosphate buffer saline. The test solution was prepared by mixing each sample with hydrogen peroxide solution. The mixture was then incubated for 10 minutes at 37˚C. After incubation the absorbance was measured at 230nm against a blank. The percentage of scavenging of hydrogen peroxide was calculated using the formula

$$\% \text{ of Scavenging} = \frac{\text{Absorbance of control} - \text{Absorbance of test} \times 100}{\text{Absorbance of control}}$$

Where,

Control – Hydrogen peroxide without sample

Blank – Phosphate buffer

Test – Sample with hydrogen peroxide

DPPH FREE RADICAL SCAVENGING ASSAY

DPPH solution was prepared by mixing with methanol. The radical scavenging activity was determined by dissolvingvarying concentrations of sample with 0.1mM methanolic DPPH. The mixture was mixed thoroughly and incubated in dark for 30 minutes. After incubation the absorbance was measured at 517nm in spectrophotometer. The percentage of DPPH free radicals scavenging activity was calculated by following equation

$$\% \text{ of Scavenging} = \frac{\text{Absorbance of control} - \text{Absorbance of test} \times 100}{\text{Absorbance of control}}$$

Where,

Blank – Methanol

Control - DPPH in methanol without sample

Test – Sample with DPPH in methanol.

ANTICOAGULANT ACTIVITY

Blood was drawn and trisodium citrate was added. It was centrifuged and pure platelet plasma was obtained. The negative control was prepared by mixing plasma, saline water and calcium chloride. The positive control was prepared by dissolving plasma, EDTA and calcium chloride. The test sample was prepared by mixing plasma, sample and calcium chloride. The test samples were kept at an angle of 45˚ and time was calculated to record the clot formation period. This time is called as Prothrombin Time.

THROMBOLYTIC ACTIVITY

Few drops blood was placed on a white tile and allowed clot. The sample was placed on the clots and time was noted down to calculate duration taken by the sample to lyse the blood clots.

ANTI-INFLAMMATORY ACTIVITY

1ml of RBC was mixed with equal volume of phosphate buffer solution. This was made up to 10ml by adding phosphate buffer solution. Sample was prepared in buffer solution. The test solution was prepared by mixing the sample in buffer of RBC suspension. This was duplicated into 2 sets. One set was kept at 54˚C in

boiling water for 20 minutes and another set was kept at 10˚C in freezer for 20 minutes. Dissolve ¼ tablet of disprin in buffer. The control was prepared by mixing this solution with RBC suspension and incubated at 54˚C for 20 minutes. After incubation the sample was centrifuged for 5 minutes. The supernatant was collected and measured at an absorbance of 540nm. The percentage of hemoglobin inhibition by the sample was calculated by the following equation

$$\% \text{ of Hemoglobin Inhibition} = 1 - \frac{OD_2 - OD_1}{OD_3 - OD_1} \times 100$$

Where
OD_1 – Absorbance of test sample unheated
OD_2 – Absorbance of test sample heated
OD_3 – Absorbance of control heated

PREPARATION OF BIOLOGICAL FILM

Sample was mixed with agar and gelatin respectively and dissolved in distilled water. The solution was mixed well and heated at 100˚C in boiling water till it forms a sticky consistency and poured on to the flat surface (tile) coated with oil and air dried.

CYTOTOXICITY ASSAY

To perform the test, VERO cells were loaded into a 96-well plate and MEM medium was added. After 24 hours of incubation, various concentrations of the sample were added to the wells. The cells were incubated for 24 hours and washed with phosphate buffer. The supernatant was removed from each well and MTT solution was added to each well and incubated for 3 hours. The supernatant was then removed and DMSO was added to dissolve the formazan crystals at room temperature for 30 minutes. The optical density of each well was measured using an ELISA reader at 570 nm using DMSO as blank. The viability of the cells for each concentration was calculated using the following formula:

$$\% \text{ of Cell Viability} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of control cells}} \times 100$$

ANTICANCER ACTIVITY

HEP2 cells were plated in 24 well plates and incubated at 37˚C with 5% CO_2 condition. After the cell reaches the confluence, media was removed from the wells and various concentrations of the sample were added and incubated for 24 hours. After incubation the sample was removed from the well and washed with phosphate buffered saline or MEM without serum. MTT was added and incubated for 24 hours. After incubation, DMSO was added in all the wells. The absorbance at 570nm was measured with ELISA reader using DMSO as the blank. Measurements were performed and the concentration required for a 50% inhibition was determined graphically. The percentage of cell viability was calculated using the following formula

$$\% \text{ of Cell viability} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of control cells}} \times 100$$

RESULTS AND DISCUSSION

CONFIRMATORY TESTS

SOLUBILITY TEST

Chitin dissolves in only certain specific solvents and acids likehexafluoroacetone**,** *N,N-dimethylacetamide* mixed with 5% lithium chloride. To check for the solubility of the substance extracted we mixed it with 70% orthophosphoric acid and hexafluroisopropanol which solubilized the extract proving that the substance is chitin

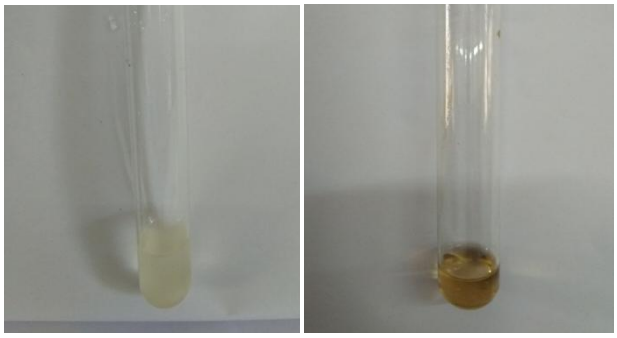

FIG 3 - SOLUBILITY IN ORTHOPHOSPHORIC ACID

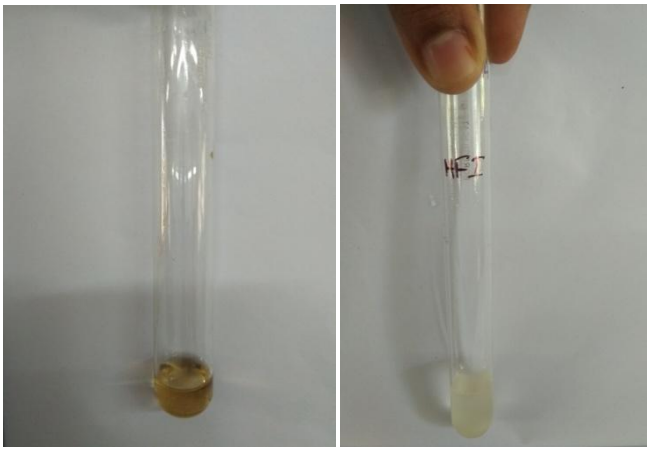

FIG 4 - SOLUBILITY IN HEXAFLUROISPOPROPANOL

THIN LAYER CHROMATOGRAPHY

Since chitin is a carbohydrate it has the ability to react with iodine fumes and change its colour to brown, this test proves that the extracted sample is chitin.

FIG 5 – TLC PLATE

CHARACTERIZATION STUDIES

FOURIER TRANSFORMATION INFRARED SPECTROSCOPY (FT-IR)

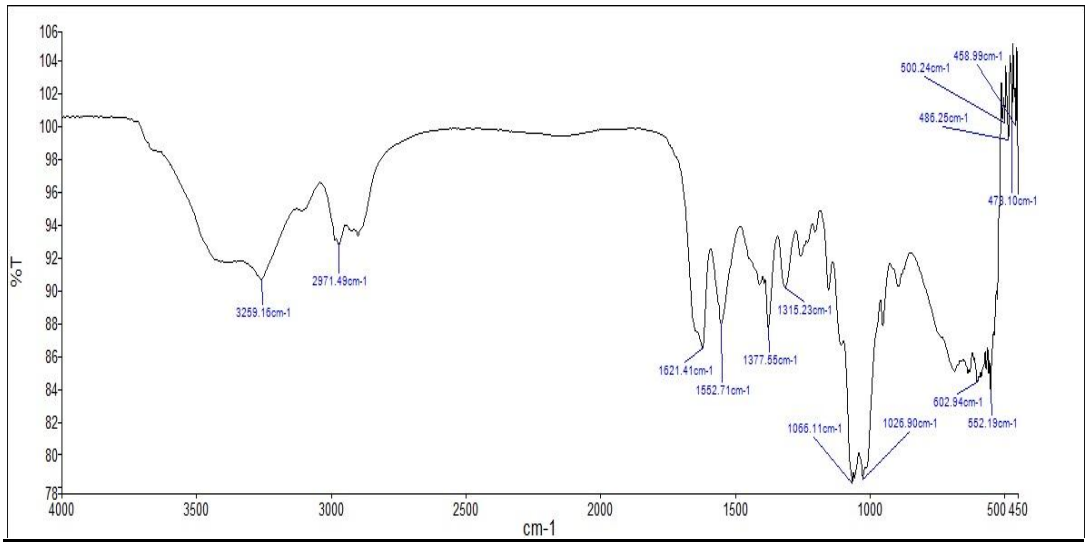

FIG 6 – FTIR results for standard chitin

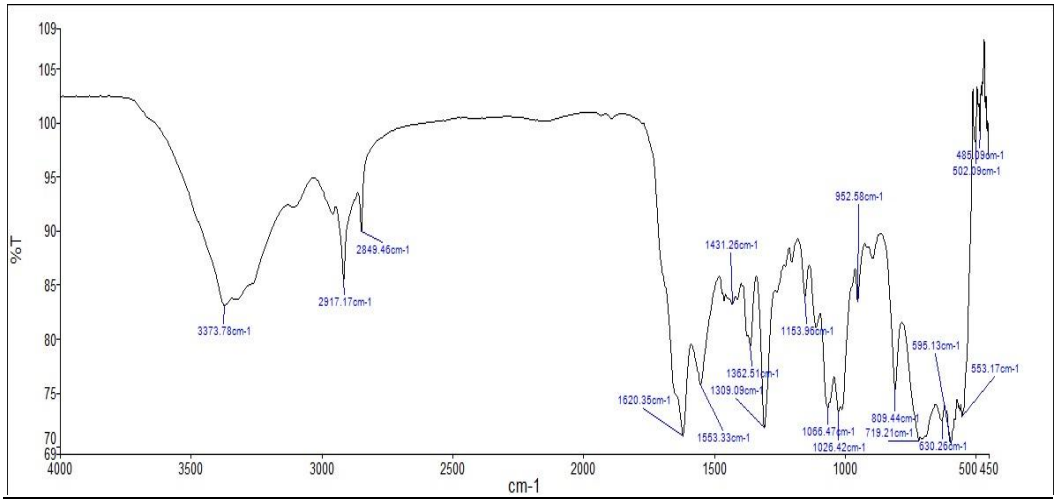

FIG 7 – FTIR results for chitin extracted from insects

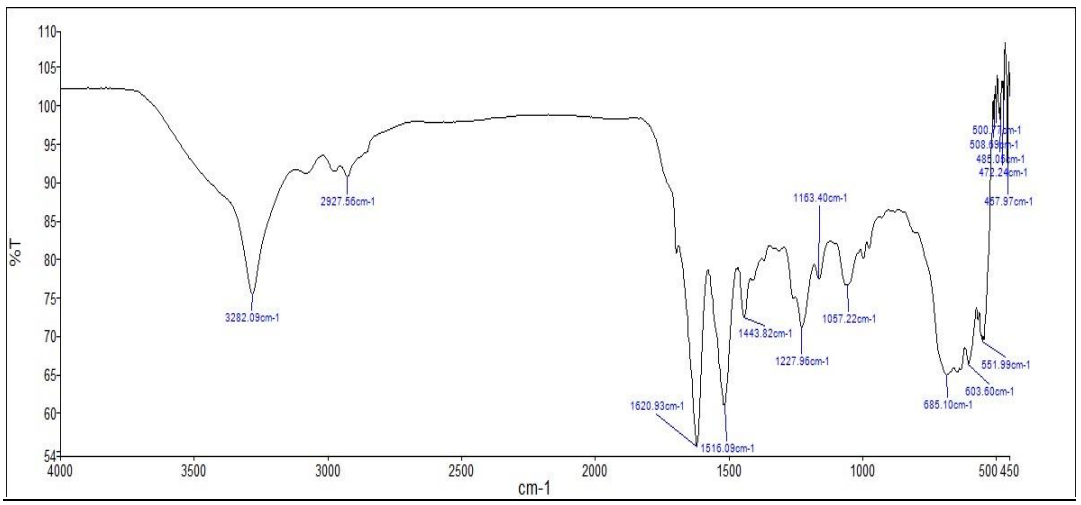

FIG 8– FTIR results for chitin extracted from silkworm cocoon

The curves present in the standard chitin were like the curves of insect and silkworm cocoon extracts tested. This proves that it was chitin from the samples which produced similar peaks to that of the standard

POWDER XRAY DIFFRACTION (PXRD)

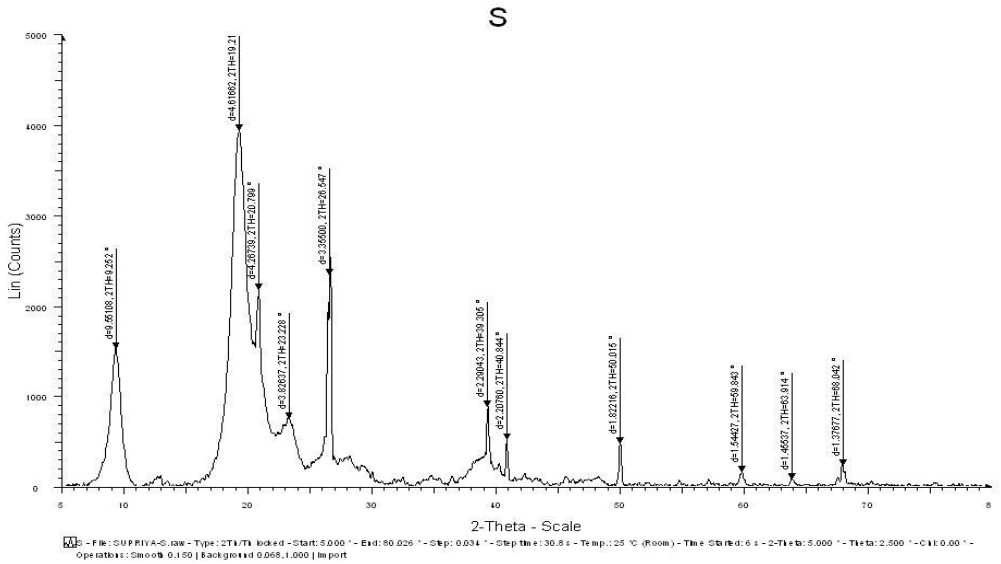

FIG 9- XRD results of standard chitin

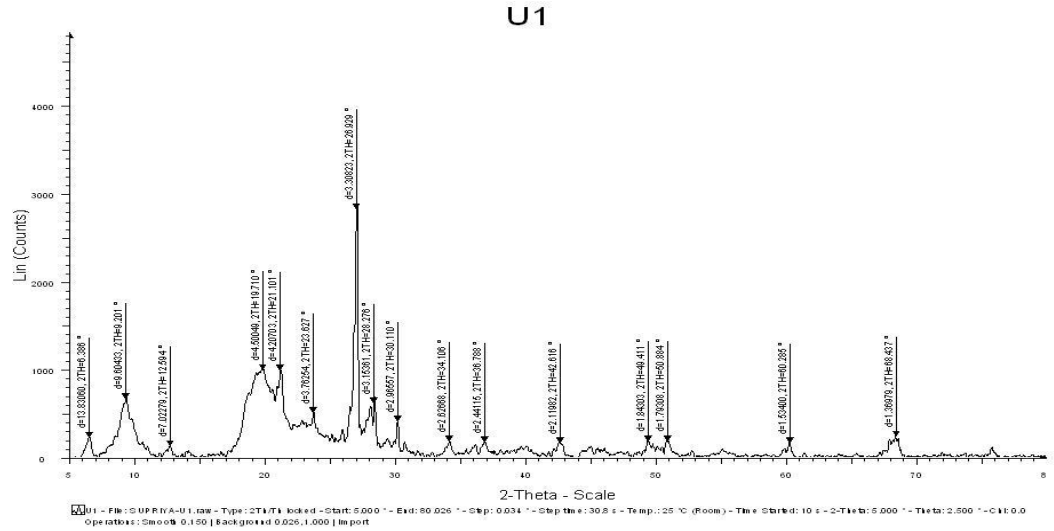

FIG 10 - XRD results of chitin extracted from insect

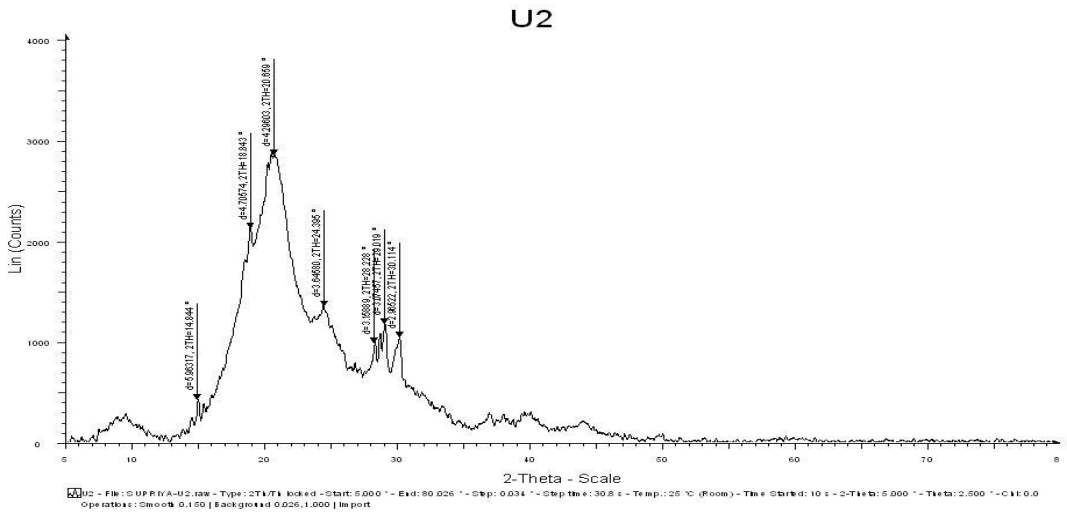

FIG 11 - XRD results of chitin extracted from silkworm cocoon

The peaks present in the standard chitin were similar to that of the peaks present in the chitin extracted from the insect and silkworm cocoon. The broadness of the peak in fig 11 shows that abundant amount of chitin is present in the silkworm cocoon.

ANTIMICROBIAL ACTIVITY

FIG 12 – ANTIBACTERIAL ACTIVITY (*Pseudomonasaeruginosa, Klebsiellaspp, E.coli, Streptococcuspneumoniae, Staphylococcusaureus*)

FIG 13 – ANTIFUNGAL ACTIVITY(*Penicilliumchrysogenum, Aspergillusflavus, Aspergillusfumigatus, Candidaalbicans, Candidatropicalis*)

ORGANISMS (BACTERIA)	ZONE OF INHIBITION		
	CONTROL	INSECT	SILKWORM COCOON
Pseudomonasaeruginosa	20mm	32mm	30mm
Klebsiellaspp	23mm	30mm	26mm
E.coli	20mm	30mm	32mm
Streptococcuspneumoniae	14mm	16mm	18mm
Staphylococcusaureus	19mm	24mm	24mm

TABLE 1 – ZONE OF INHIBITION

ORGANISMS (FUNGI)	ZONE OF INHIBITION		
	CONTROL	INSECT	SILKWORM COCOON
Penicilliumchrysogenum	15mm	22mm	20mm
Candidaalbicans	34mm	33mm	36mm
Aspergillusflavus	10mm	15mm	10mm
Candidatropicalis	32mm	38mm	35mm

Aspergillusfumigatus	6mm	15mm	9mm

TABLE 2 – ZONE OF INHIBITION

The diameter of different inhibition zones were measured and compared. The size of the inhibition zone of the samples were greater than the zone of control proving that the extracted chitin has antimicrobial properties.

ANTIOXIDANT ACTIVITY
HYDROGEN PEROXIDE RADICAL SCAVENGING ASSAY

INSECT	SILKWORM COCOON
26.88	23.44
22.58	23.01

TABLE 3 - PERCENTAGE OF H_2O_2 SCAVENGING

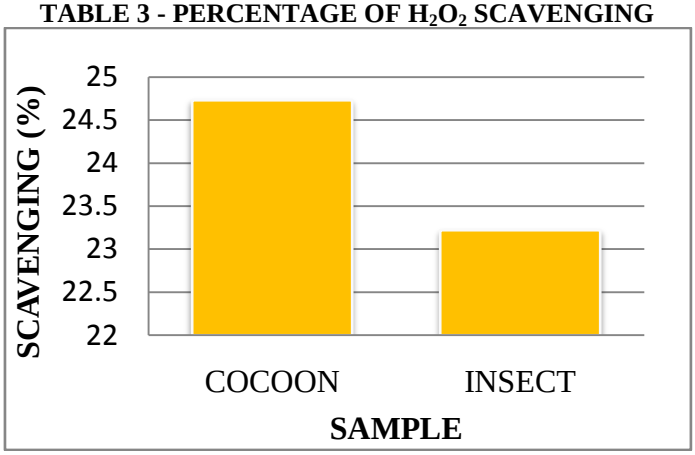

FIG 14 - GRAPH SHOWING H_2O_2 SCAVENGING ACTIVITY

The anti oxidant activity chitin obtained from silkworm cocoon was 24.73% and insect was 23.22% and proved that the extracted chitin has good anti oxidant property

DPPH FREE RADICAL SCAVENGING ASSAY

CONCENTRATION (µl)	% OF SCAVENGING
10	15.5
20	17.5
30	23.5

TABLE 4 – PERCENTAGE OF DPPH SCAVENGING ACTIVITY OF SILKWORM COCOON CHITIN

CONCENTRATION (µl)	% OF SCAVENGING
10	**18**
20	**15.5**
23	**29**

TABLE 5 – PERCENTAGE OF DPPH SCAVENGING ACTIVITY OF INSECT CHITIN

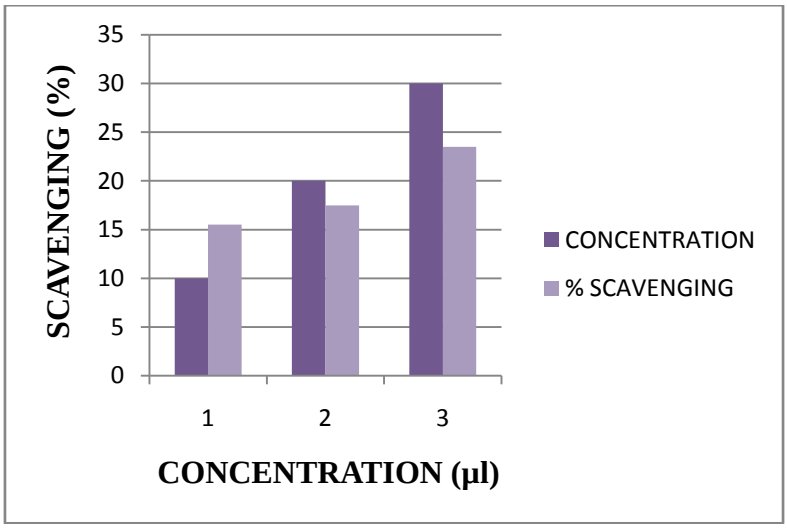

FIG 15 – GRAPH SHOWING DPPH SCAVENGING ACTIVITY OF SILKWORM COCOON CHITIN

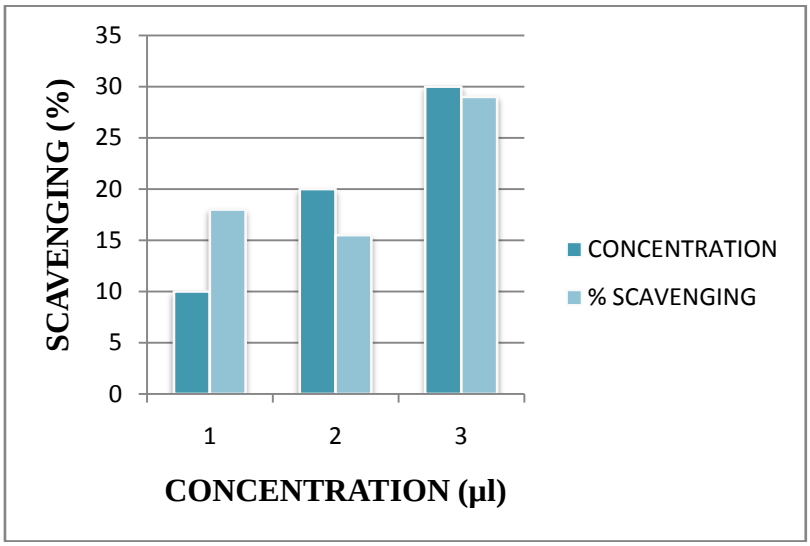

FIG 16 – GRAPH SHOWING DPPH SCAVENGING ACTIVITY OF CHITIN FROM INSECT

The anti oxidant property was found to be 18.83% for cocoon and 20.83% for insects .

ANTICOAGULANT ACTIVITY

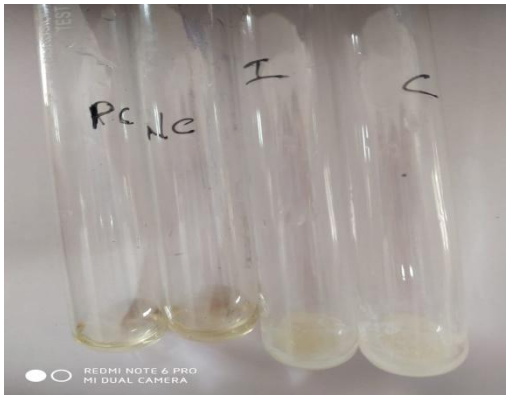

FIG 17 – ANTICOAGULANT TEST

No formation of clots are found even after a time period 30 minutes. This proves that the extracted chitin has non coagulating factors present in it.

THROMBOLYTIC ACTIVITY

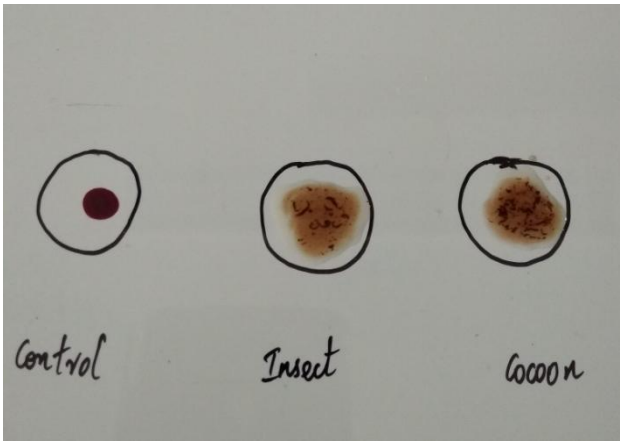

FIG 18 – THROMBOLYSIS TEST

The extracted chitin was able to lyse the blood clots within time duration of 10 minutes. This proves the presence of thrombolytic property present in the extracted chitin.

ANTI-INFLAMMATORY ACTIVITY

INSECT	SILKWORM COCOON
27	33.11

TABLE 6 – ANTI INFLAMMATORY TEST

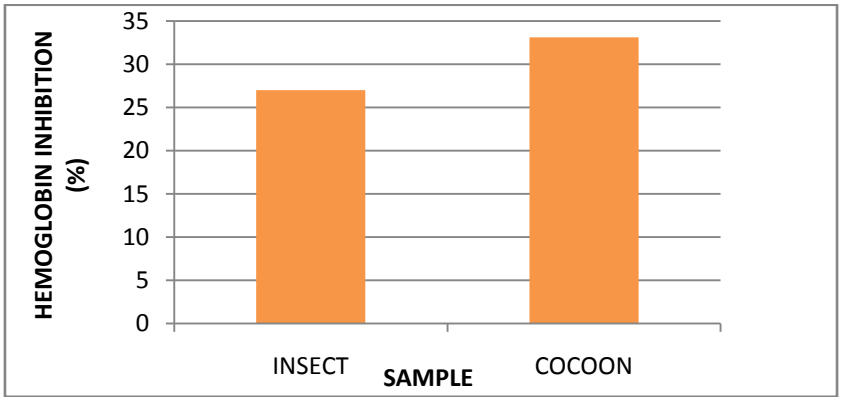

FIG 19 – GRAPH SHOWING ANTI INFLAMMATORY TEST

The extracted chitin from insects and silkworm cocoon has shown to have good hemaglobin inhibiton about 27% and 33.11% for insects and cocoon respectively, which showed the presence of anti inflammatory property in them.

PREPARATION OF BIOLOGICAL FILM

FIG 20 – GELATIN FILM

FIG 21 - AGAR FILM

The biological film is prepared using gelatin and agar as a binding agent.

CYTOTOXICITY ASSAY

CONCENTRATION (µl)	%VIABLE
10	**174.30**
20	**188.26**
30	**121.22**
40	**168.71**
50	**199.44**

TABLE 7 – CYTOTOXICITY TEST OF CHITIN FROM COCOON

CONCENTRATION (µl)	% VIABLE
10	**115.64**
20	**12.0.67**
30	**143.65**
40	**169.52**
50	**174.86**

TABLE 8 – CYTOTOXICITY TEST OF CHITIN FROM INSECT

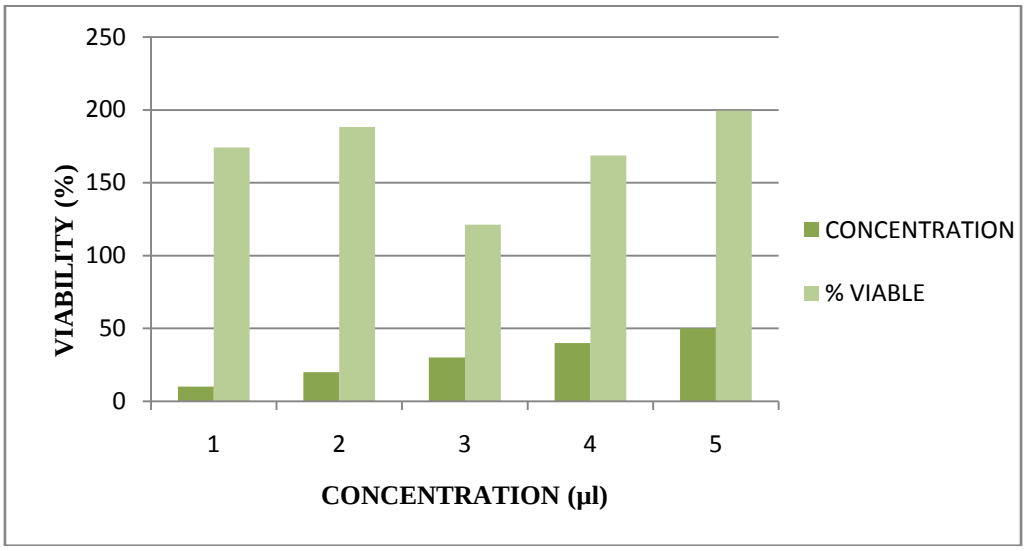

FIG 22 – GRAPH SHOWING CYTOTOXICITY TEST OF CHITIN FROM COCOON

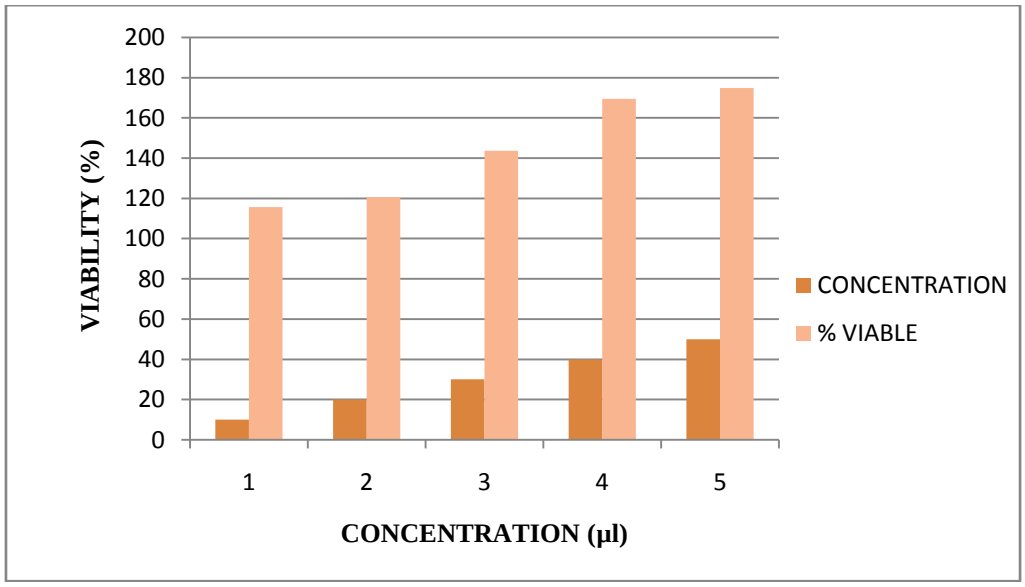

FIG 23 – GRAPH SHOWING CYTOTOXICITY TEST OF CHITIN FROM INSECTS

The percentage viability of cells is more than 90% which shows that the obtained chitin has good cell activation property required for proliferation of the cells.

ANTICANCER ACTIVITY

CONCENTRATION (µl)	% VIABLE
10	40.8
20	22.43

TABLE 9 – ANTICANCER TEST OF CHITIN FROM COCOON

CONCENTRATION (µl)	% VIABLE
10	31.78
20	28.01

TABLE 10 – ANTICANCER TEST OF CHITIN FROM INSECTS

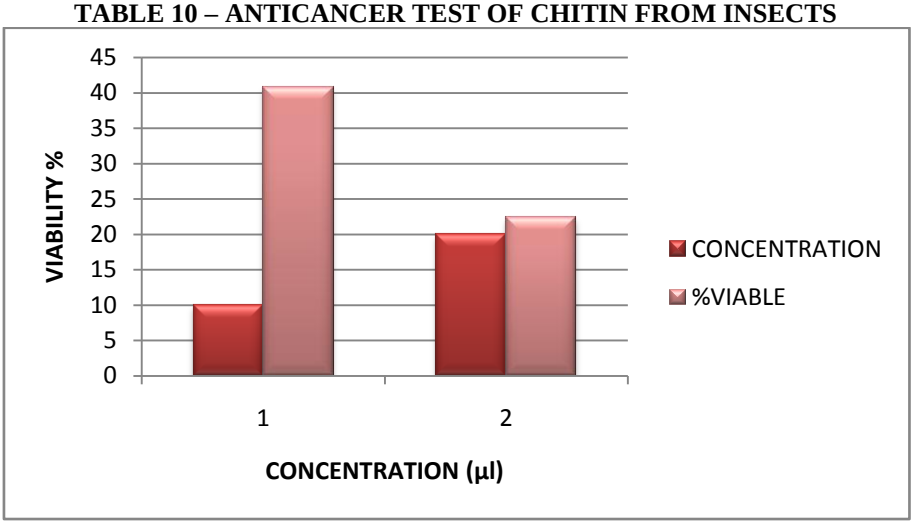

FIG 24 – GRAPH SHOWING ANTICANCER TEST OF CHITIN FROM COCOON

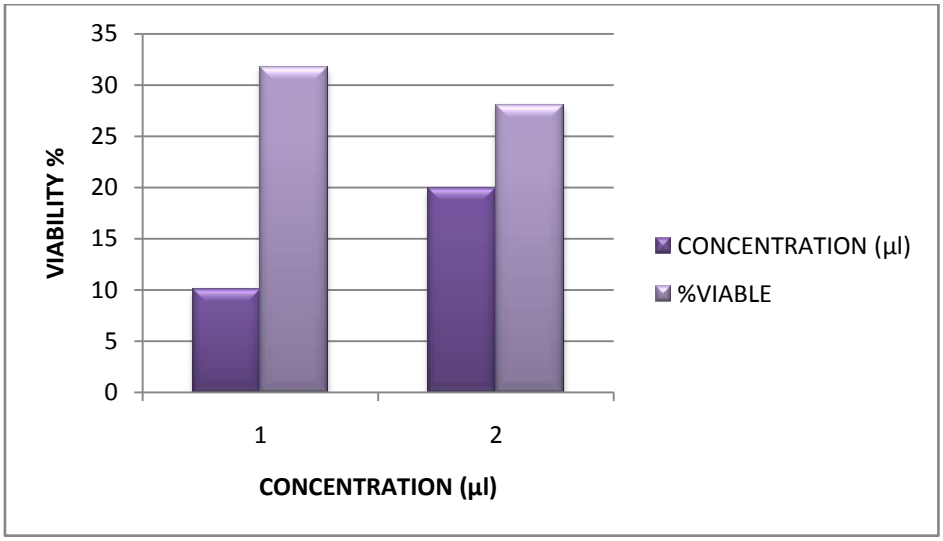

FIG 25 – GRAPH SHOWING ANTICANCER TEST OF CHITIN FROM INSECTS

The percentage viability of sample treated cancer cells were found to be less than the untreated control cells. This shows that the chitin obtained from insect and cocoon has good anticancer property.

III. Conclusion

Chitin, a polysaccharide having various significant properties has been isolated from the insects (winged termites) and silkworm cocoon. The extracted chitin has been tested by the solubility test and thin layer chromatography. Its configuration has been established by the characterization studies such as FTIR which shows the curves of the extracted chitin being similar to the curves of the standard. The Powder XRD study peformed confirmed its structure by illustrating the peaks analogous to that of the standard chitin and the broadness of the peak shows the abundance of chitin present in the sample. The obtained chitin was then tested to learn about its various therapetuic and industrial properties. The antimicrobial activity performed proved that the sample have properties inhibitng the growth of the microbes. The anti-oxidant test done proves it has good scavenging activity. Anti -coagulant activity performed shows it has less amount of blood clotting factors in it.

The thrombolytic activity done for the extracted chitin was able to lyse the blood clots. The obtained chitin was used for the preparation of biological films using agar and gelatin as binding agent which can be used for various industrial and therapeutic applications. The anti inflammatory test performed showed good hemoglobin inhibition in insect and cocoon chitin which proves that it has good anti inflammatory property. The acquired chitin was used for the preparation of biological film using agar and gelatin as a binding agent which can be used for various therapeutic and industrial purposes. Cytotoxicity assay performed proved that the acquired chitin is nontoxic and has cell proliferative properties since the viability of the sample treated cells were greater than the control cells. The anticancer test done showed the inhibition of the growth of cancer cells when compared to the untreated control cells.

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