Isolation and Characterization of Protein derived from Garlic Scale and their application in Bone Tissue Engineering

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2015

NATIONAL INSTITUTE OF TECHNOLOGY

ROURKELA

CERTIFICATE

This is to certify that the thesis entitled *"***Isolation and Characterisation of protein derived from Garlic Scale and their application in Bone tissue Engineering***"* submitted by **Ms. Ankita Kundra** [Roll No. 111bt0530] in partial fulfilment of the requirements for the award of the degree of Bachelor of Technology in Biotechnology at National Institute of Technology, Rourkela is an authentic work carried out by her under my guidance.

To the best of my knowledge the matter embodied in the thesis has not been submitted to any other University/Institute for the award of any degree or diploma.

Date: 10 May, 2015

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ABSTRACT

In this project, we aim to find out the protein in garlic scale that could be used as a growth factor for bone cell proliferation. Garlic is one of the most researched bulbs and has immense medicinal values. The aqueous solvent of garlic scale was subjected for ammonium sulphate precipitation followed by dialysis to extract out the protein. The protein was concentrated using lyophilization. SDS-PAGE confirmed the presence of a protein of molecular weight 25 kDa approximately. Using the CD Spectroscopy analysis, the percentage of alpha helixes and beta sheets were found out. Fourier Transform Infra-red Spectroscopy suggested the presence of protein by showing some characteristics peaks. The endothermic peak for this new formulation shows a broad region of water evaporation. At every concentration, the samples showed a very positive effect on the MG63 cell viability by a considerable increase in the cell proliferation. Thus, protein from garlic scale can be used as a biological response modifier for tissue engineering applications.

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INTRODUCTION

Regardless of the advances in all fields of medicine, organ transplantation suffers numerous flaws. There is a potential risk of immune rejection that is coupled with a huge number of side effects as drugs trade off patient's natural immune responses to pathogens and grafts of allogeneic sources. Thus, it will make them more susceptible to infection. The acute shortage of organ donation and rising requirement for organ donors highlights for the need of novel approach.

Cell transplantation is another alternative treatment to whole organ transplantation. During the creation of an autologous implant, donor tissue is harvested and separated into individual cells. These cells are then adhered and cultured onto a proper substrate that is ultimately implanted at the site of the functioning tissue. However, it is believed that isolated cells cannot form new tissues by them. Most cells of the primary organs are believed to be anchorage-dependent and require proper environments and require the presence of a supporting material. Thus the development of suitable substrates for both in-vivo and in-vitro tissue culture is very essential to the success of any cell transplantation.

This led to the advancement of tissue engineering with an aim to replace the worn and damaged parts that are bio-compatible and functional with the body. Tissue engineering can perhaps be best defined as the use of a combination of cells, engineering materials, and suitable biochemical factors to improve or replace biological functions. Scaffold, cells and growth-stimulating signals are referred to as triads of tissue engineering. Scaffolds are the three dimensional porous matrix that mimics body's own extra-cellular matrix. These acts a platform onto which cells can attach, multiply, migrate and began synthesizing new tissue. ECM provides a structural support and growth inducing environment for the cells to adhere,

grow, migrate and respond to the signal. It may also act as reservoir of many growth factors and potentiates its bioactivities. A major approach has been to convert the synthetic polymer to bioactive scaffolds by proper impregnation of the proteins throughout the scaffolds. Many proteins have been preferred due to their biocompatibility nature and their ability to support cell attachment, growth proliferation. The most commonly used proteins are collagen and silk protein, but they have their drawbacks that limit its use in tissue engineering. Collagen has the risk of contamination by the virus since it is derived from animal sources. Apart from this, it is difficult obtain collagen with consistent properties and has poor wet strength. Silk protein has long degradation rates and causes inflammatory reactions. Thus, efforts have been recently dedicated to the development of novel strategies for specific biological factors that would initiate the recruitment of cells and its differentiation with much safety. Various proteins such as colostrums, BMP and keratin have been discovered which act as growth factors for such applications and many more are being screened for potential use. These proteins stimulate cell adhesion, proliferation, migration and differentiation. The present research focuses on the development of suitable constructs that can be used tissue engineering applications.

Through this project, we are aiming to find out the proteins in garlic scale that can be further utilized in tissue engineered constructs. The scale do not possess toxicity, and hence their chemical composition can be found out with much safety.

Garlic scientifically named as *Allium sativum*, is the species in the onion species, *Allium*. It is utilized for both culinary and restorative purposes in view of its odour and flavour. Garlic and its concentrates have been utilized to treat diseases since time immemorial. It contains an abundance of chemical compounds that have been demonstrated to have advantageous impacts to secure against diseases. It contains at least 33 Sulphur compounds, 17 amino acids, several enzymes and minerals such as selenium. Dried, powdered garlic contain

approximately 1% allium. When fresh garlic is crushed, it releases a compound called alliin. Injury to the garlic activates the enzyme allinase that converts allium to allicin. Allicin is further metabolized to vinyldithiines [1]. Garlic has truly been utilized to treat wounds, extreme loose bowels, clogging and parasitic contaminations, and heart diseases and many other healths related disorders [2]. Garlic shows hypolipidemic, hypoglycemic, cardioprotective, immunostimulant and pro circulatory impacts. Thus, garlic plays an invaluable role in the prevention and therapy of major diseases.

In contrast, garlic skins or peels being inedible part of garlic, has not been studied for their health benefits. There are a few reports on the chemical composition of garlic skins. Little research has been done on garlic scale that can prove to be fruitful in regulating cell's physiological properties.

LITERATURE REVIEW

Garlic:

Garlic is renowned throughout the centuries for being the spice food item and boasts a large number of preventive and health giving properties. Sulphur containing compounds such as alliin and allicin helps in lowering blood pressure and cholesterol levels [3]. These are active constituents of garlic and are rapidly absorbed and metabolized [2]. Moreover, the consumption of garlic has been proposed as a therapy for prevention against cancer development. These are the organo-sulphur compounds that are responsible for its chemopreventive activity [4].

Garlics have been known to show some immunomodulatory effects. Some the protein showing these effects were extracted from garlic which have been recognized as lectins or agglutinins ASA I and ASA II [5].They have mitogenic activity and have a numerous potential in therapeutics immunomodulation [6]. Immune dysfunction may be caused by certain diseases. Thus, modification of immune system by garlic can be used in prevention of certain diseases beforehand [7].An extract of aged garlic have been reported to have number of pharmacological effects, including immunomodulation.

Layers of garlic cloves:

Each clove of garlic are surrounded by a layer of protective sheaths. Going from outward to inward, the first leaf of a lateral bud is a layer of dry protective skin since it has the toughened lignified surface that protects the soft storage leaf that it surrounds. The next layer, the second leaf of the lateral bud that constitute the bulk of the clove called the storage sheet

which as the high content of stored food. Next is the leaf three, the sprout leaf consisting of entirely of sheaths [8].

However, normally during the development of lateral bud the first and the second leaf may form dry protective skin and the third leaf sheath swells to from the storage leaf and sometimes the third leaf may develop a little which remain enclosed to the storage sheet. The scale have been known to possess anti-oxidants [9].

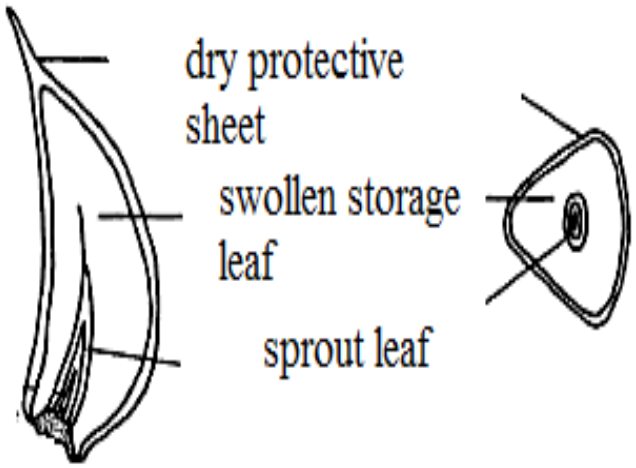


Figure 1: Outline drawing of median longitudinal cross-section of garlic clove

Proteins in garlic that regulate it's behaviour:

Proteins from various natural resources have been known to possess proteins that assess cell viability. Proteins present in garlic have been shown to produce various anti-modulatory effects, the major being Allium sativum agglutinins ASA I and ASA II (25 kDa). While ASA I is a dimeric protein comprising of lectins subunits of 12.5 and 13.0 kDa and ASA II is a homodimer comprising of lectins polypeptide of 12kDa [10]. Both lectins have high specificity for mannose and have hemagglutination property.They exhibit mitogenic activities towards lymphocytes, thymocytes and human peripheral blood [11].

Apart from these proteins, high molecular weight agglutinin ASA (110 kDa), the anti-microbial protein alliumin (13 kDa), the antifungal protein allivin (13 kDa) , from round-clove garlic have also been discovered. ASA110 is a glycoprotein of two identical subunits of 47 kDa. ASA 110 has been found to have a high content of aspartic acid, leucine and serine but the low content of methionine and cysteine [12]. Two major proteins of 40 kDa and 14 kDa have also been separated. While 14kDa protein was localized in the cortical cells (CC), 40KDa protein was present in the parenchyma sheath cells (PSC) of garlic bulbs, which was revealed by immune-cytochemical and ultra-structural study [13].

Therapeutic effects of garlic:

The benefits of fresh garlic have been affirmed for different cardiovascular risk factors, particularly its capacity to lessen blood cholesterol levels, repress aggregation of blood platelets and break down clots [14]

The mode of action for chemopreventive property is still unknown, but various mechanism have been proposed [15]. The mechanisms include the effects on drug metabolizing enzyme, tumour growth inhibition and antioxidant properties [16]. Garlic is rich in antioxidant phytochemicals that includes organo-sulphur compounds and additionally flavonoids, for example, allixin, which are equipped for scavenging free radicals [17]. Garlic additionally contains selenium, needed for ideal function of the antioxidant enzyme glutathione peroxidase.

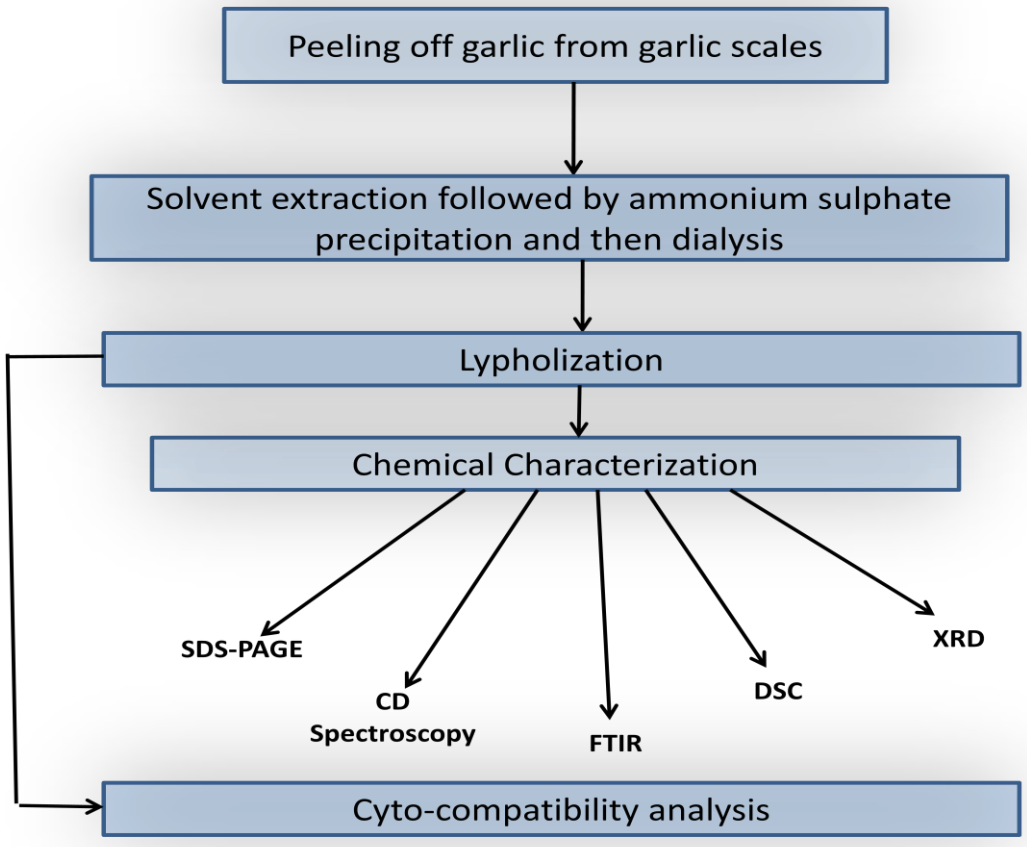
A good deal of evidence suggests beneficial impacts of the regular dietary intake of garlic on mild hypertension. Its blood pressure lowering properties is linked to allicin content and hydrogen sulphide production [18]. Allicin is known to have systemic vasodilation and angiotension inhibiting effects [19]. Various garlic extracts have known to effective against the wide spectrum of bacteria including species of Escherichia, Salmonella, Staphylococcus,

Streptococcus etc owing to high allicin content [14]. Studies have demonstrated that high concentrates of garlic extracts possess fungistatic and fungicidal activity both in vitro and in vivo. Administration of raw garlic orally protects the tissue against oxidative stress through its antioxidant property. Hence, garlic can be treated as a potent drug for the treatment of alcoholic disorders [20]. Alcoholic extract derived from garlic has an anthelminthic activity [21]. Because of numerous scientific literature available, garlic antibiotic, anti-diabetic and anti-cancer effects are accepted all over the world but its anti-coagulant, anti-inflammatory and immunomodulatory requires more attention of researchers

OBJECTIVE

- Chemical Characterization of proteins from garlic scale
- Effects on cell viability by MTT assay

WORK PLAN



MATERIALS AND METHODS:

MATERIALS:

Dulbecco's Modified Eagle Medium (DMEM), Trypsin-EDTA solution, Foetal Bovine Serum, Antibiotic-Antimycotic solution, 12 well plate, acrylamide, bis-acrylamide, Ammonium persulphate, Tetramethylethylenediamine, sodium dodecyl sulphate , ammonium sulphate and MTT assay kit were purchased from Hi-Media, Mumbai, India and MG63 cells were obtained from NCCS, Pune.

METHODS:

Removal and purification of garlic scale:

Garlic was purchased from the market and using a knife, the scale were peeled off carefully. After collecting a substantial amount of garlic scale, the scale were washed thoroughly with distilled water thrice. After washing, they were spread on a tray. The tray was covered with a polyethylene wrap that was punched with holes so that sufficient air reaches the scale and does not allow the scale to escape since it is light in weight.

Preparation of samples:

The garlic scale were coarsely grounded and weighed. It was then added to water and left for aqueous extraction for the next 24 hours. For every 1 gram of powdered garlic scale, 40 ml of water was taken. The solution was then filtered using a filter paper [22].

Ammonium Sulphate Precipitation:

The solution was taken in the beaker and stirred using magnetic stirrer. Using the ammonium sulphate precipitation table, to get 100% saturation equivalent amount of salt to be added for the given solution was calculated. While the sample was stirring, a known amount of salt was added slowly. The beaker was then moved overnight at 4°C.

Dialysis of the precipitated protein:

The precipitate was collected by centrifugation at 10,000g for 20 minutes. The precipitate was then dissolved in water. A dialysis membrane was cut into desired length and dipped in warm water. Clips were used to close one end of the tube. The protein sample was pipette and another end was tied. The tube was kept in dialysis buffer i.e. water. The water war stirred with a magnetic stirrer for at least 3-4 hours. A fresh volume of water was replaced after every 4 hours. The above step was done overnight.

Protein concentration using Lyophilization:

The Dialyzed Protein was then kept at -20°C overnight and taken for lyophilisation the next day. In the lyophilizer, the temperature was set at -50˚C for 36 hours and the samples were taken out [23].

Separation and Identification of proteins using SDS-PAGE:

The protein isolated from the sample was separated using the standard protocol [24].

Resolving gel:

The casting frames were set on casting stands. The gel solution was prepared and was swirled thoroughly. An appropriate amount of separating gel solution was pipetted out into the gap between the glass plates. To make the top of the gel horizontal, water was filled into the gap until an overflow. The gel was allowed to gelate for 20-30 minutes.

Stacking gel:

Water was discarded and the stacking gel was pipetted until an overflow. The well forming comb was inserted without trapping air under the teeth. It was allowed to gelate for 20-30 minutes.

Marker protein:

Page Ruler Plus Prestained Protein Ladder:

The glass plates were taken out of the casting frame and set in the cell buffer dam. The running buffer was poured into the chamber and the comb was then taken out from the stacked gel. Sample buffer was prepared and lypophilized garlic extract was mixed with it. The protein marker was the loaded in into the first and the last well. The prepared sample was loaded properly into the wells. The electrodes were connected. The whole setup was run with approximately 65 voltages. The SDS Page was stopped when the last sign of the protein marker reached the foot line of the glass plate.

Staining:

The gel was placed in 100 ml of CBB R250 solution (0.1% CBB R250/40% methanol/10% Glacial Acetic acid) and agitated on an orbital shaker or rocking platform for 2-4 hours.

De-staining:

The gel was placed in 100 ml of destaining solution (10% methanol/7% Glacial Acetic Acid) and agitated on an orbital shaker until resolved blue bands and a clear background appear.

CD spectroscopy Analysis:

To determine the percentage of the alpha helix and beta sheets, the sample (both lyophilized and dialyzed protein) was taken for CD spectroscopy analysis.

There were a total of 5 samples which was taken.

Sample 1: dialyzed protein after day 1

Sample 2: dialyzed protein after day 2

Sample 3: lyophilized sample

Sample 4: lysozyme 0.1mg/ml (control)

Sample 5: lysozyme 1 mg/ml (control)

Fourier Transformed Infra-red (FTIR) Spectroscopy:

FTIR analysis was done to determine the functional groups present in the samples. It was performed using an Alpha Bruker FTIR Spectrophotometer instrument with the scanning range of 400 cm^{-1} -4000cm^{-1}.

The sample was prepared and 3-5 mg was placed on the spectrophotometer plate and adding a drop of solvent over it. Lysozyme was used as a control.

Differential Scanning Calorimetry:

It is used in the thermal analysis in which the difference in amount of heat required to increase the temperature of the sample and reference is measured as a function of temperature. Here both sample and reference are taken at the same temperature. It was performed using DSC 200 F3, Maia Netzsch. 5 mg of the sample was taken and the change in the heat flow was 0.1-10 mW. The temperature was varied from 20-200 ° C.

X-Ray Diffraction:

XRD is non- destructive analytical technique that is used to predict the crystal information about the material. It is based on scattering intensity of X-Ray light by the atoms of a crystal for generating diffraction pattern to produce interference.3-5 mg of the sample was taken for XRD analysis using Expert High Score X-ray Diffractometer.

Cell Viability Test:

Subculturing of MG63 cells:

Biosafety cabinet was wiped thoroughly with 70% ethanol. Media (DMEM+10% decomplemented FBS+2% anti-toxin antimycotic arrangement), PBS bottle and Trypsin-EDTA were kept inside the hood after wiping with 70% ethanol. UV light was switched on for 30 minutes and the blower rate was situated at 5. More than 70 % Confluent T25 flask was taken out from CO_2 incubator and kept on the working bench. With the help of sterile pipette, used media was removed and 2 ml of PBS was added for proper washing. 2 ml of Trypsin-EDTA was added to uproot the cells adhered to the surface. The flask was then kept in the CO_2 incubator for 4-5 minutes. The flask was then taken out and tapped so that all the cells come out in the suspension. Again 2 ml of fresh media was added. The whole cell suspension was centrifuged at 1000 rpm for 5 minutes in a 15 ml falcon tube. After discarding the supernatant, the cell pellet was resuspended in 1 ml of fresh media. 100 µl of cell suspension were counted using haemocytometer (Trypan blue dye exclusion test) [25].

Preparation of different sample concentrations:

10 mg of the lyophilized sample was taken and mixed with 10 ml of distilled water to prepare 1mg/ml sample concentration. Again from this sample, 1 ml was taken and mixed with 9 ml distilled water to obtain 100µg/ml concentration.

From this sample, 1ml was taken and mixed with 9 ml of distilled water to prepare 10µg/ml of the sample. Thus, three different sample concentrations were made and 100µg/ml and 10µg/ml were used for analysis.

Seeding of MG63 cells:

$5 * 10^4$ cells were seeded into each well of a 24 well plate. 300 µl of DMEM media was added to each well. The cells were cultured in an incubator at 37˚C and 5% CO_2 for one day. 1

mg/ml, 100µg/ml and 10µg/ml were added to the wells. Wells without any sample were taken as control.

MTT Assay:

After a day of cell seeding, 20 µl of the MTT reagent was added to each well. Cells were incubated in an incubator at 37˚C and 5% CO_2 for 3 hours. The media was removed then and 200 µl DMSO was added to dissolve the purple colour formazan crystals formed after the incubation. The optical density was measured at 595 nm [26].

RESULTS AND DISCUSSION

Isolation of Protein from garlic Scale:

Sl no.	Procedure	Starting material	Yield	Colour	Appearance	Solubility
1	Cold extraction and filtration	10gm (crude)	400ml	Pale yellow	Transparent filtrate	Insoluble
2	Ammonium sulphate precipitation (100%)and dialysis	300ml	10ml	brown	Turbid solution	Soluble
3	**Ammonium sulphate precipitation (100%) and dialysis and lyophilization**	**300ml**	**150mg**	**Light brown**	**Dry powder**	**Partially Soluble**
4	Ammonium sulphate precipitation (80%) and dialysis and lyophilization	100ml	24mg	Light brown	Dry powder	Partially Soluble
5	Ammonium sulphate precipitation (60%) and dialysis and lyophilization	100ml	15mg	Light brown	Dry powder	Partially Soluble
6	Ammonium sulphate precipitation (40%) and dialysis and lyophilization	100ml	5mg	Light brown	Dry powder	Partially Soluble

Table 1: Composition, yield and physical appearance of garlic scale extracts

Separation of protein using SDS-PAGE:

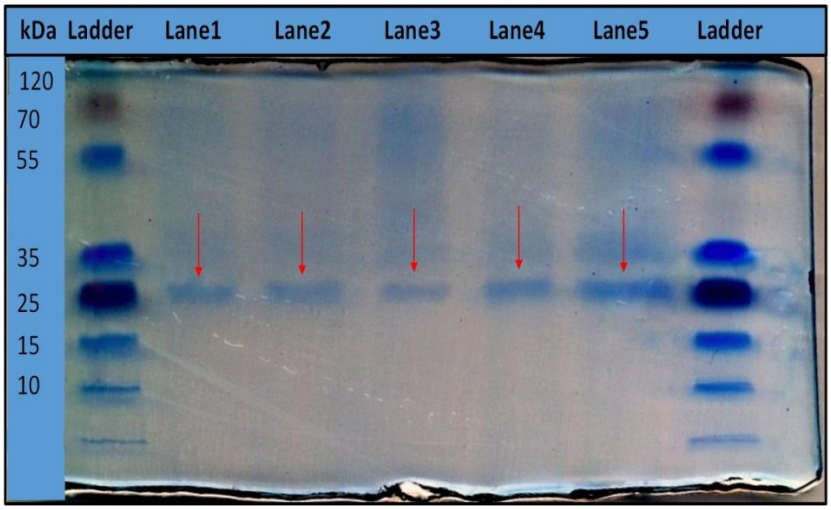


Figure 2: SDS-PAGE of garlic scale extracts: Lane 1,2&3: Crude cold extract. Lane 4&5: Lyophilized sample

The figure shows the SDS PAGE of the lyophilized garlic extract. All lanes showed presence of protein in both crude cold extract and lyophilized samples. Lane 1,2&3 showed less intense band compared to the lyophilized protein sample band in lane 4 & 5. The protein present in the sample was found to have a molecular weight of approximately 25 KDa. Experiments were conducted to study the effect of garlic extracts on the cell growth and proliferation.

CD Spectroscopic analysis of garlic scale extracts:

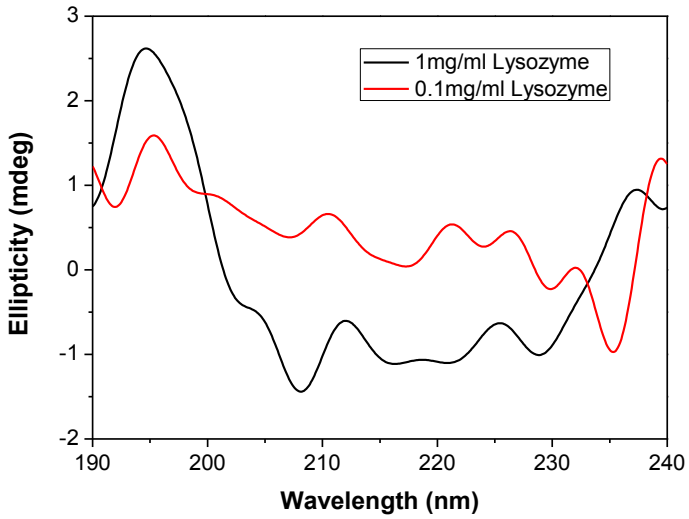


Figure 3: CD spectra of Lysozyme as a control parameter

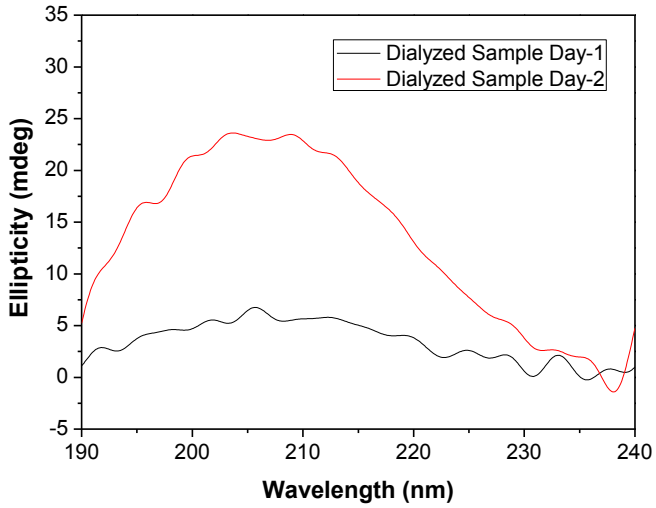


Figure 4: CD spectra of Dialyzed Aqueous Protein Solution

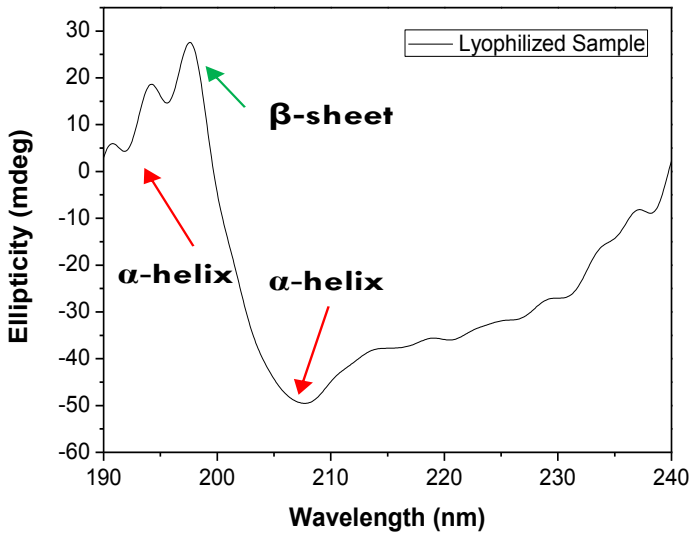


Figure 5: CD spectra of lyophilized garlic scale extracts

The secondary structure of a protein is determined using CD spectrum in the far-UV region. The CD spectrum of lysozyme of concentration 1mg/ml clearly indicates that there are more percentages of alpha helixes with negative peak around 208 nm and positive peak at 190 nm. The CD spectrum for the lyophilized sample shows a 207.6 nm in the negative region and a positive peak at 191.1 nm that shows that there is more percentage of the alpha helix. It also has the peak at 197.7nm in the positive region which is the characteristic peak of beta sheets. Thus we can say that the new formulation has more percentage of alpha helix as compared to beta sheets.

Whereas the CD spectrum for the dialyzed sample gave a distorted curve from which we can say that dialyzed protein may have degraded during the dialysis process showing no specific characteristics peaks of the secondary structures.

FTIR analysis of garlic scale extracts:

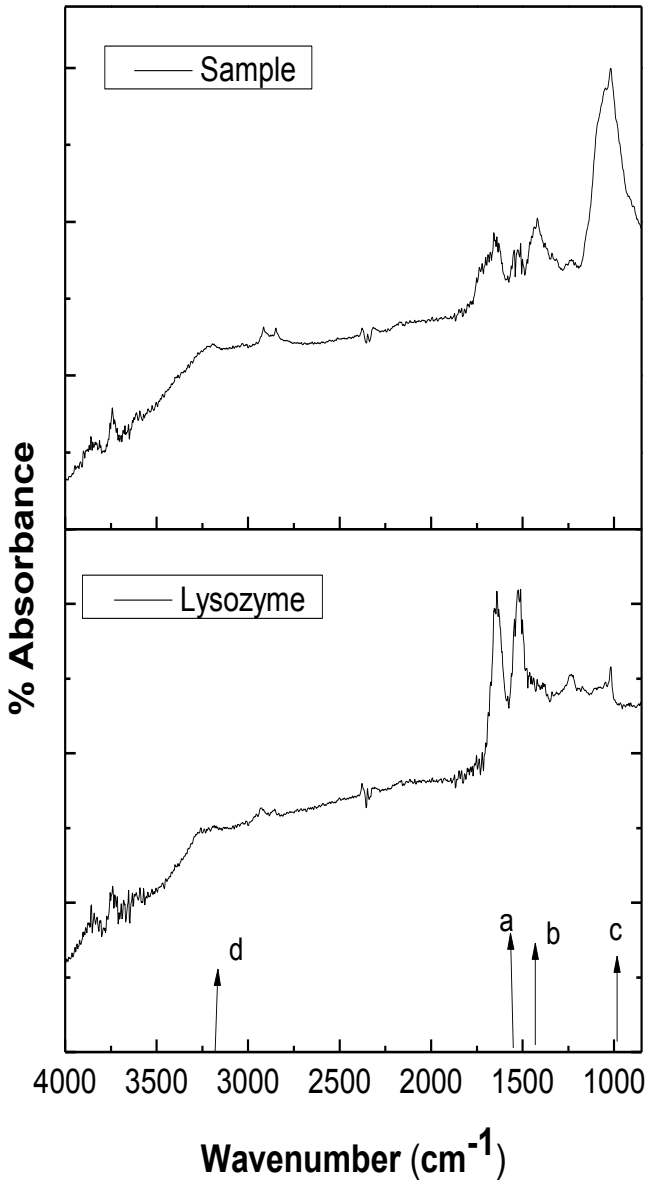


Figure 6: FTIR analysis for both Lysozyme and lyophilized garlic scale extracts

In protein, the polypeptide repeat unit provide nine infra-red absorption bands namely amide A, B and I-VII. For the protein backbone, amide I and II are the most prominent vibrational bands.

The Amide I is mainly due to the presence of protein secondary structural components and lies mostly in the range of 1700-1600 cm ¯1 which is entirely due to C=O stretch of the peptide linkages. The amide band derives from in-plane NH bending and the CN stretching. Other amide bands are very complex depending on the force field, the nature of side chains and hydrogen bonding, which therefore are of little practical use in the protein conformational studies.

The Amide I is highly sensitive to hydrogen bonding pattern and small variation in geometry which makes it useful in characterising protein's secondary structures. It gives rise to C=O bond due to hydrogen bonding and unique geometry and a little N-H bending.

a	(1650-1640 cm $^{-1}$) Amide-II region corresponding to C=N stretching
b	(1520-1507 cm $^{-1}$) Amide-I region corresponding to N-H deformation and C-N stretching
c	1045cm^{-1} corresponds to C-O stretching vibrations
d	3000cm^{-1} corresponds to O-H stretching vibrations

Table 1: Band assignment for the Lysozyme

Absorption peaks(/cm)	Inference
1660 cm^{-1}	Vibration absorption of amide I group
1522 and 1499 cm^{-1}	Amide II region of C=N stretching
1023 cm^{-1}	C-O stretching vibrations
3004 cm^{-1}	O-H stretching vibrations

Table 2: Band assignment for the Extracted protein

DSC analysis of garlic scale extracts:

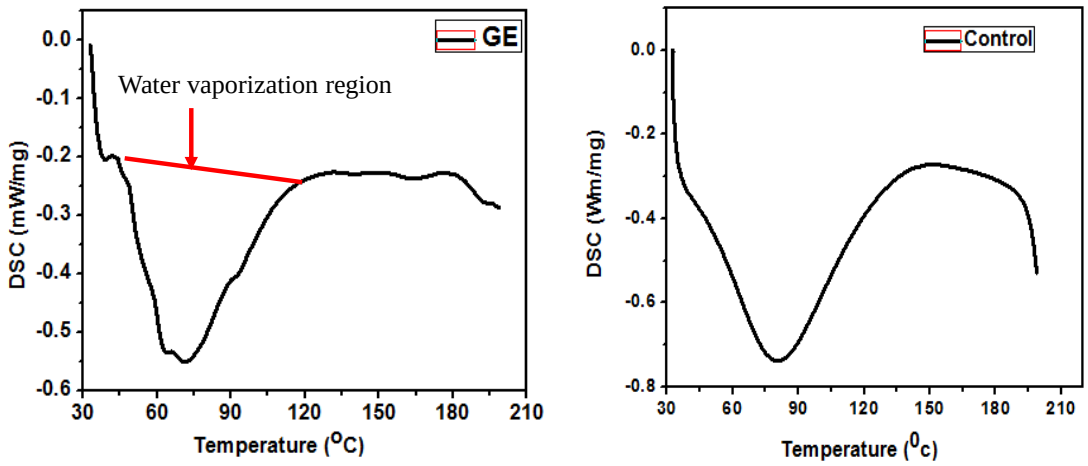


Figure 7: DSC analysis of Lysozyme and lyophilized garlic scale extract

Formulations	$T_{onset,m}$	T_m	$T_{e,m}$	Area under the curve	Enthalpy (ΔH)	Entropy (ΔS)
	(°C)	(°C)	(°C)		J/g	J/g/°C
Control	39.33	126.28	81	239.6	239.6	121.38
Garlic extract	44.04	113.12	70	143.6	143.5	59.143

Table 3: Values of temperatures, Enthalpy and Entropy in DSC analysis

The endothermic peaks of both control and garlic extract showed a broad region of evaporation of water in a range of 60-100 °C. Peak temperature of the control is nearly at 81°C but the peak temperature for the garlic scale extract shifted to lower regions of 70° C. If we make a depth analysis to the endothermic graph of the extract, we will find there are two endothermic peaks are available nearly at 70°C. The second one can be explained as the shoulder peak to the initial endothermic peak. The area under the curve of the both

endothermic graphs explained the enthalpy of the formulations. As per the literature more the area under the curve, more is the enthalpy. Thus in turn, enthalpy of the control is more than the sample. Entropy of the system is also calculated from the endothermic of the formulations which explain that the garlic scale extract has a lower entropy value than the control.

X- Ray Diffraction analysis of lyophilized garlic scale extracts:

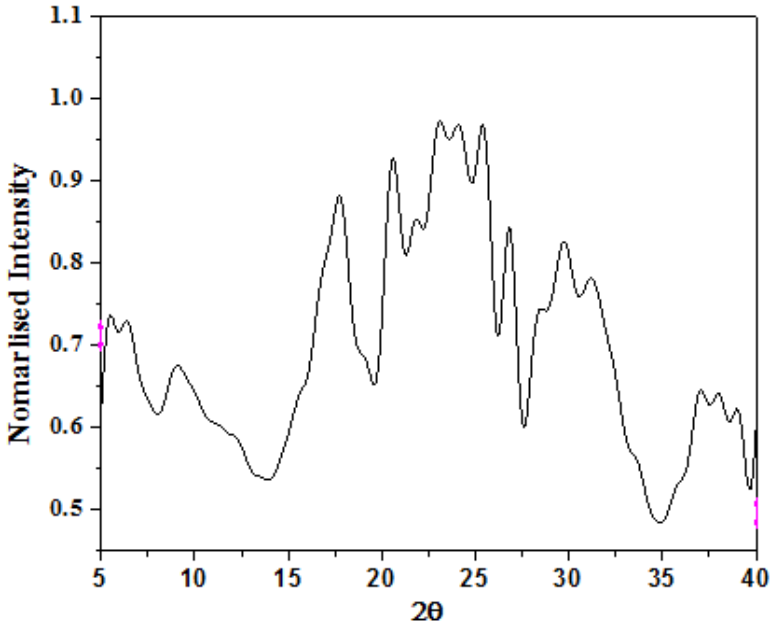


Figure 8: XRD analysis of lyophilized garlic scale extracts

The graph shows X-ray diffraction for the lyophilized garlic extract. It shows the maximum peaks at 23°, 25.46°, 20.3°, 17.68°, 24.1°, 20.9°. The minimum peak was around 21.7°. All the blunt peaks are in the region of polymer and were found to be amorphous.

Since there is no reference present with respect to the garlic extract, a more detailed account is required for a clear analysis for this new formulation.

Cell viability by MTT assay:

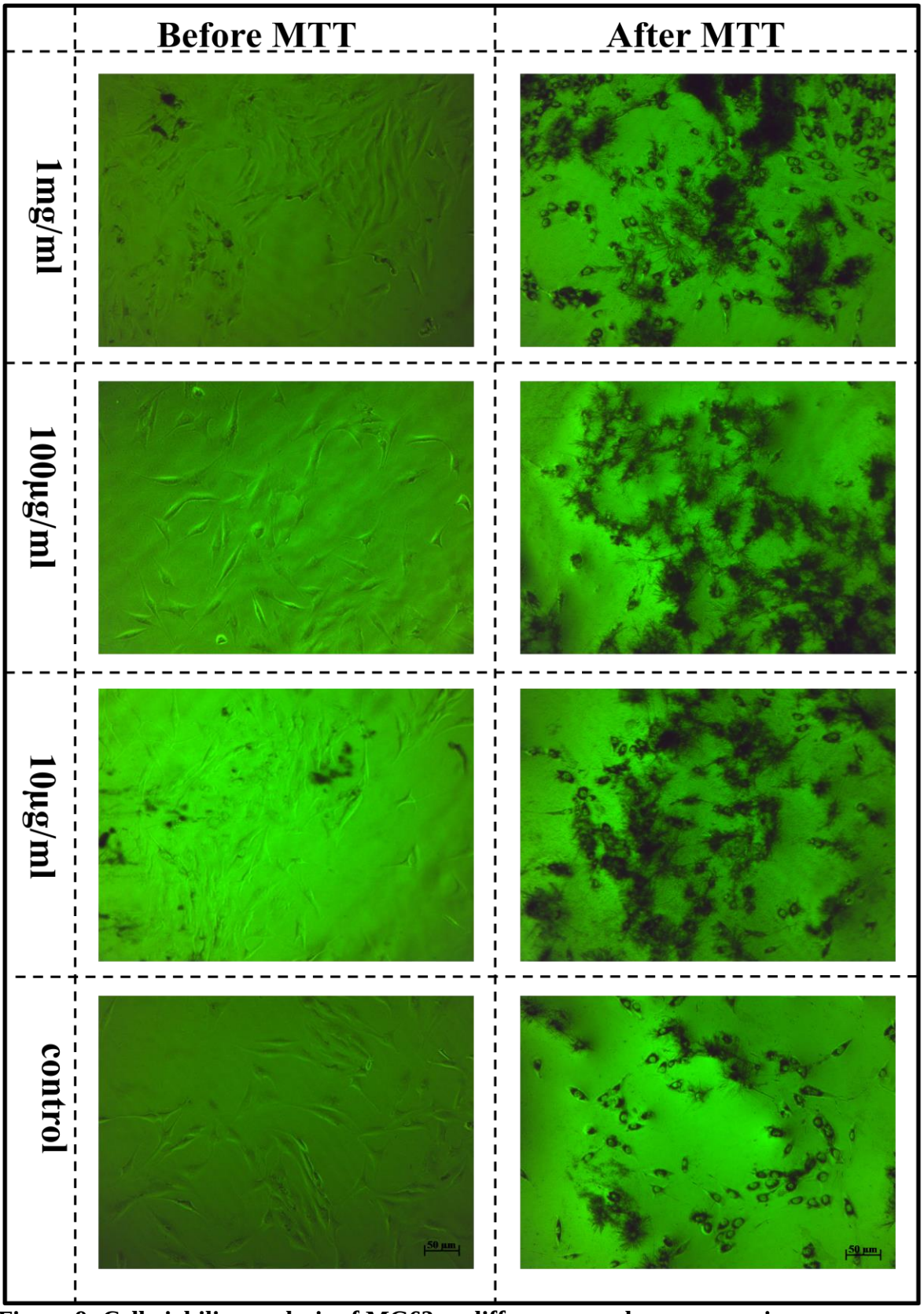


Figure 9: Cell viability analysis of MG63 at different sample concentration

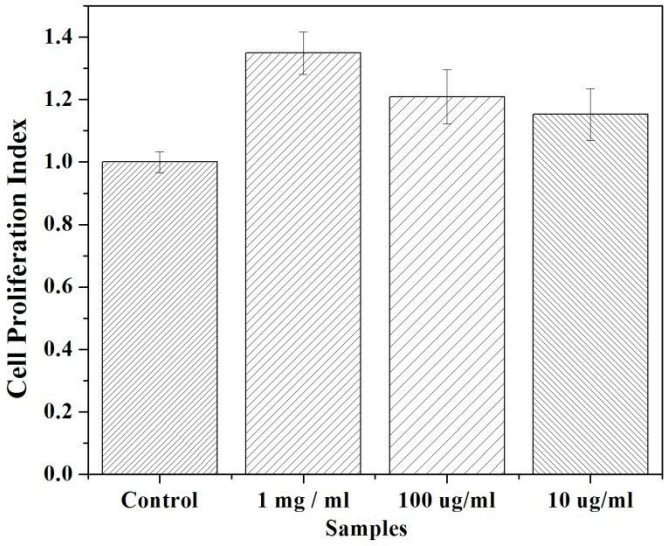


Figure 10: Study of cell proliferation index by MTT assay. Experiments were done in triplicate.

The cell viability with the addition of different sample concentration was studied. It was observed that for 1mg/ml, the cell showed maximum proliferative index (1.4 fold increase as compared to control).

CONCLUSION

Proteins extracted from garlic scale were chemically characterised and their effects were checked on cell viability. SDS-PAGE suggested the presence of proteins whose secondary structure was later determined by CD Spectroscopy. Fourier Transform Infra-Red identified the functional group that clearly elucidated the presence of proteins. The endothermic peak in the DSC explained the broad range of water evaporation for this new formulation. The study of cell proliferation indicated that garlic scale can be used modifier for further application in tissue engineering.

The research is at the preliminary stage and a detailed account and characterisation is required for the better understanding of the project to make it useful for potential applications.

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