Chemical Characterization of Active Components derived from Garlic Scales and their Potential Applications in Tissue Engineering

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ROURKELA

C E R T I F I C A T E

This is to certify that the thesis entitled *"**Chemical Characterization of active components derived from Garlic Scales and their potential applications in Tissue Engineering**"* submitted by **Ms. Shambhavi** [Roll No. 110BT0597] 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: 10th May 2014.

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A C K N O W L E D G E M E N T

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ABSTRACT

To overcome the drawbacks of conventional organ transplants, a new approach has been developed to replace damaged tissues with tissue engineered construct. For this purpose, it is important to regulate cell adhesion, migration and proliferation in order to achieve a substantial percentage of therapeutic efficacy. Garlic is one of the most researched bulbs owing to its high medicinal value. Garlic scales have been chemically characterized by extraction to observe their effects on cell physiology. UV-Visible characterization of the scales suggested presence of proteins which was confirmed later using SDS PAGE. Aqueous solvent gave a significant amount of extracts so it was used for all the further experiments. Using Fourier Transform Infra-Red spectroscopy, presence of some functional groups were confirmed out of which thiol group suggested traces of allicin in the scales which is present largely in the bulk garlic. Proteins from various sources act as cell growth modulators so the protein from the scales was tested for cell viability and antimicrobial activity. Proteins were concentrated using lyophilisation. Three different cell lines namely HaCaT, HeLa and MG63 were used. Three sample concentrations were prepared: 1mg/ml, 100µg/ml and 10µg/ml. The samples didn't show any antimicrobial activity inferring either they lack antimicrobial properties or the concentration of the samples is too low to cause any effect. The sample having concentration 10µg/ml enhanced cell adhesion of MG63 cells considerably. Migration studies were conducted which varied for different cell lines at different concentrations. Samples showed a very positive effect on the HeLa cell viability at every concentration by a considerable increase in the cell proliferation. In conclusion, active components from garlic scales can be used as a biological response modifier for tissue engineering applications.

CONTENTS

List of Figures

List of Tables

CHAPTER 1

INTRODUCTION

INTRODUCTION

Conventional organ transplantations face serious drawbacks like shortage of organ donors and immune rejection. With the use of potent immunosuppressive agents, patients' susceptibility to infections and cancer has increased. These transplantations are also associated with pathogens like lymphocytic chloriomeningitis virus[1]. To overcome such drawbacks, a new approach has been developed in the field of tissue engineering to replace tissues with serious damages with an aim to improve the biocompatibility and bio functionality.

Tissue engineering makes use of three basic tools which are cells, scaffolds and growth factors. Various proteins 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, migration and differentiation. Some of these proteins are as follows:

Source	**Protein**
Milk	Colostrum
Gin Seng root	Ginsenoside
Platelets	Platelet derived growth factor
Human bone	BMP
Human hair	Keratin

Table 1: Cell growth modulatory proteins

In this project, we tried to find out the proteins present in the garlic scales and to study their properties to enhance or suppress cell adhesion, proliferation and migration.

Allium Sativum L. commonly known as garlic is one of the best researched and exploited herbal remedies. It is important for the culinary values of its odour and flavour. It is very frequently used in food as a spice. Garlic contains enzymes like allinase, sulphur-containing compounds like alliin, and compounds like allicin produced enzymatically from alliin. Conventionally, it has been used to treat wounds, infections, diabetes, diarrhoea, rheumatism, heart diseases and many other health related disorders[2]. Experimental studies have demonstrated that garlic exhibits antibacterial, antilipidaemic, anticarcinogenic, immunostimulant, antihypertensive, cardioprotective and hypoglycaemic properties. Clinically, garlic has been investigated for a large variety of clinical indications, namely, hypertension, hypercholesterolaemia, diabetes and also for the prevention of arteriosclerosis and cancer. It has also shown antiinflammation, immunomodulation, antimicrobial and antifungal properties[3].

Garlic has many therapeutic uses as mentioned above, so the scales possess a potential to be used for tissue engineering applications without facing the disadvantage of immune rejection.It is the outer protective covering of garlic. Much research has been done on the bulk garlic but garlic scales haven’t grabbed much attention in the research area.

There seems to be a very little literature about the toxicity of widely used garlic scale, which makes it an important thing to investigate chemical composition of garlic scales and their safety related to human handlers and consumers. Phytochemical screening and elemental analysis of garlic scales and toxicological effects of its aqueous extract should investigated well.

CHAPTER 2

LITERATURE REVIEW

LITERATURE REVIEW

2.1. Garlic

Garlic has been used since ancient times as a medicinal plant to cure various disease conditions in humans. Its basic medicinal uses are lowering blood pressure and cholesterol, prevent cancer and fight infections. Sulphur containing compounds are its active constituents and are rapidly absorbed and metabolized[4]. Studies have suggested that garlic helps in lowering the total cholesterol concentrations by about 10%. It alters the HDL/LDL ratios[5]. Garlic acts as a mild hypertensive by lowering blood pressure. Garlic causes the inhibition of platelet aggregation and enhances the fibrinolytic activity by reducing clots on a damaged endothelium. Its important use is an antidiabetic. By various mechanisms, it controls the blood sugar level. Various animal data and in vitro studies suggest that garlic is used for the prevention of solid tumors[6].

2.2 Composition of Garlic

Garlic is known to have atleast 33 sulphur compounds, 17 amino acids, several enzymes and minerals like Selenium. Out of all the Allium species, it contains the highest concentration of sulphur compounds. These compounds are responsible for its medicinal effects and the pungent odour[2]. Dried and powdered garlic contains 1% Alliin. When the garlic is crushed or cut, the injury activates the enzyme Allinase, which converts Alliin to Allicin. Allicin is metabolized further to vindylithiines. This breakdown takes hours at room temperature and occurs in minutes during cooking[7]. Allicin has microbial effects. It acts against many viruses, fungi, bacteria and parasites. Aged garlic or garlic oil contain a variety of products of allicin transformation but crushed garlic has much more physiological activity[8].

2.3 Therapeutic effects of Garlic

Hydrogen sulphide production in garlic is responsible for its blood pressure reducing properties. This property is also attributed to the allicin content liberated from alliin. Allinase has vasoldilating and angiotensin inhibiting effects[9]. Angiogenesis helps in wound healing. Various techniques have been used to determine the effects of aged garlic on re-epithelialization, wound closure and angiogenesis. A hyperglycemic patient can be treated with garlic. Garlic oil contains a variety of allyl sulphide constituents which have the same effect as glibenclamide. It is also effective in serum glucose levels. The organosulfur compounds present in garlic have a chemopreventive activity[9]. They are effective on drug metabolizing enzymes and cause tumor growth inhibition[6]. Garlic prevents the onset and development of atherosclerosis. It suppresses lipid synthesis and causes inhibition of biosynthesis of cholesteryl esters[10]. Mycobacterium tuberculosis is sensitive to garlic. Crushed garlic cloves show antibacterial activity. Garlic has an antifungal potential. It is used in the treatment of cryptococcal meningitis. Various proteins present in garlic show immunomodulatory potential. They exhibit mitogenic activities towards lymphocytes, thymocytes and human peripheral blood[11]. Some organosulphur compounds show antioxidant potential and protect against lipid related oxidations. Compounds derived from garlic regulate cytokine production and leukocyte cell proliferation. Alcoholic extract derived from garlic shows anthelminthic activity[12]. Garlic is effective in exposing dysentery and acts as a vermifuge. It is a therapeutic and prophylactic agent. It can be considered a potent drug for treating alcoholic disorders[13].

Garlic has a lot of potential to be used for medicinal purposes. It is a possibility that the scales may also contain a variety of phytochemicals which may possess many useful properties. It is a completely novel topic

2.4 Proteins which regulate cell behavior

Many proteins from various natural resources are known to possess properties which help in cell viability and show antimicrobial activity.

The protein ginsenoside is found exclusively in the plant genus ginseng commonly known as Panax. It acts as an antiproliferative agent for adipose tissue cells[14] but enhances the cell differentiation for osteoblast and osteoclast[15]. It has been seen that it has antiageing, antineoplastic, immunologic function enhancing and other pharmacological actions[16]. The inductive differentiation effect can be due to the comprehensive effect of increment in the intracellular cAMP and the inducing interferon. Since GSL has some important actions, if it could be used as a differentiation inducer combined with other antineoplastic drugs, then it would show co-antineoplastic actions in other aspects. It induces differentiation of HL-60 cells into granulocytes and also modulates protein kinase C isoforms during differentiation[17].

Colostrum is also known as beestings or first milk. Human and bovine colostrum are thick, sticky and yellowish in colour. It contains a large number of secretory immunoglobulins that helps in protecting the mucous membrane of lungs throat and intestines of the infant. It stimulates proliferation of human enterocytes like HT29[18]. Another major whey protein known as lactoferrin has been reported to accelerate bone formation by stimulating the proliferation and differentiation of osteoblasts and by apoptosis inhibition. A combination of EGF and lactoferrin helps in DNA synthesis of rat intestinal cells (IEC-6). Lactoferrin suppresses adipogenic differentiation in human hepatocarcinoma and 3T3-L1 cell lines[19].

Keratin obtained from human hair has been proposed as an appropriate protein for cell cultivation scaffolds. Yamauchi's group demonstrated the incremented growth and adherence rates of L929 fibroblasts onto keratin in comparison to collagen and glass[20]. In addition, it

was also demonstrated that the attachment and proliferation of NIH3T3 mice fibroblast cells on keratin sponges and a report was made on the cultivation and differentiation of mouse preosteoblast MC3T3-E1 cells on keratin-hydroxyapatite hybrid sponges[21].

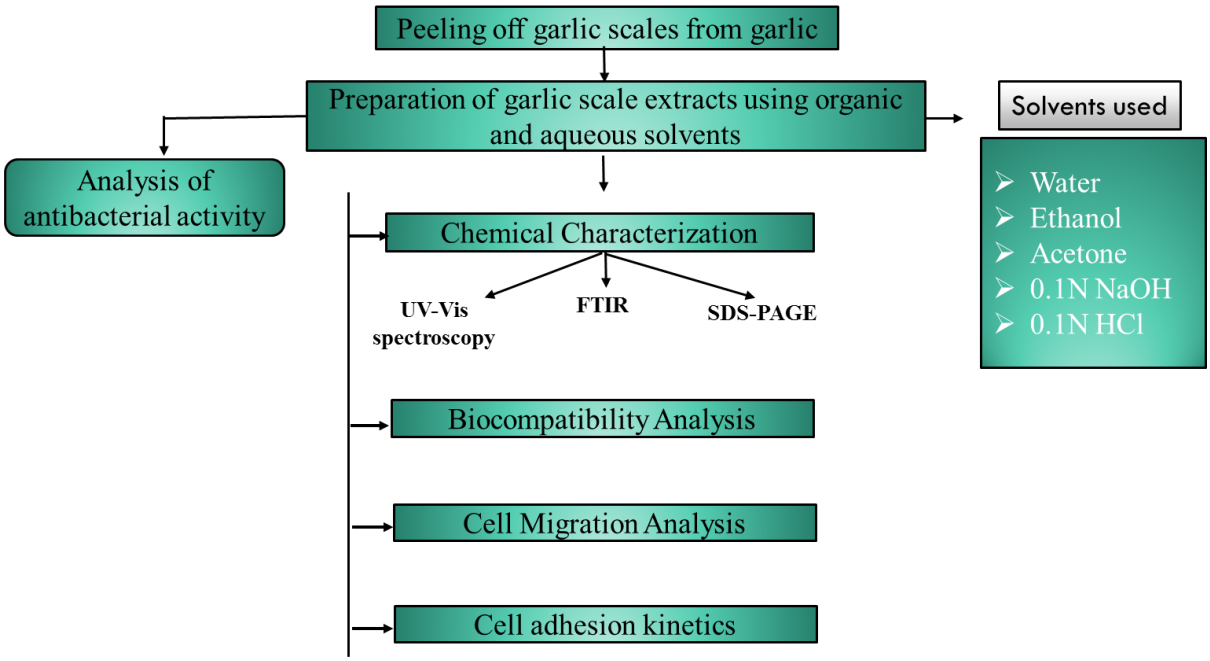
2.5 Bulb scales

Scales of other bulbs have shown properties which prove beneficial to our health. One of such bulbs is onion. It also belongs to the Allium genus. They have sulphur as the main constituent. They contain antioxidant and anticarcinogenic phytochemicals. Cartenoids and chlorophylls have been gaining attention because of their health attributes. Red onion scales contain a large amount of flavonoids which are responsible for the immune enhancement, antioxidant activity and anticancer property[22].

OBJECTIVE

- Chemical characterization of active components from garlic scales
- Effect of garlic scale extracts on cell physiology

WORKPLAN



CHAPTER 3

MATERIALS & METHODS

3.1 MATERIALS

Dulbecco's Modified Eagle Medium (DMEM), Minimum Essential Medium (MEM) Dulbecco's Phosphate Buffer Saline (DPBS), Trypsin-EDTA solution, Foetal Bovine serum, Antibiotic-Antimycotic solution, 12 well plate, acrylamide, bis-acrylamide, Ammonium persulphate, Tetramethylethylenediamine, sodium dodecyl sulphate and MTT assay kit were purchased from Hi-Media, Mumbai, India. The HaCaT, HeLa and MG63 cell lines were procured from NCCS, Pune, India.

3.2 METHODS

3.2.1 Removal and purification of garlic scales:

Garlic cloves were taken and using a knife, the scales were peeled off. The scales were washed thoroughly with distilled water thrice after a substantial amount of scales were collected. After washing, they were spread over a large area in a tray. The tray was covered with a polyethylene wrap with holes punched in it so that sufficient air reaches the scales but being light in weight, do not escape from the tray.

3.2.2 Preparation of different samples:

The coarsely powdered garlic scales were weighed and the total content was divided into five 50 ml falcon tubes. Total weight of garlic obtained was 5g.Weight of garlic scales in each sample was 1 g.

5 different solutions were taken for different samples as follows:

1. Sample 1 – Distilled Water
2. Sample 2 – 90% Ethanol
3. Sample 3 – Acetone (absolute)
4. Sample 4 – 0.1 N NaOH
5. Sample 5 – 0.1 N HCl

40 ml of these solutions were taken into each falcon tube and 1g of garlic scales were added to each tube. The solutions were allowed to stand for the next 24 hours after which they were filtered out in new falcon tubes using filter papers. The scales were stored separately for further use[23].

3.2.3 Antimicrobial assay:

Freshly prepared nutrient agar medium was sterilised and poured into petriplates under aseptic conditions and was allowed to solidify completely. 100 µl of E.Coli fresh culture was added to the plates and spread using an L-shaped rod. Three wells were punctured into each plate using a gel puncturing machine. 100 µl of the aqueous sample was poured into one of the wells. Soframycin was used as the positive control and distilled water as the negative control in the other two wells. The plates were kept in an incubator overnight and observed the next day[24].

3.2.4 Protein concentration using Lyophilisation:

The scales were peeled off from the garlic and were washed thoroughly using distilled water. They were dried overnight in an incubator at 37˚C. On complete drying, they were grinded and powdered. The coarsely powdered scales were mixed in 50 ml of distilled water. It was left to

stand for 24 hours after which the supernatant was filtered out in a new falcon tube. The volume was evenly distributed in five 50 ml falcon tubes. The tubes were kept at -20˚C overnight and taken for lyophilisation the next day. In the lyophilizer, temperature was set at -50˚C for 36 hours and the samples were taken out[25].

3.2.5 Separation and Identification of proteins using SDS PAGE:

Separation of proteins isolated from sample was performed in reference to standard protocol[26].

Resolving gel:

The casting frames were set on casting stands. The gel solution was prepared and was swirled thoroughly. 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 Pre-stained Protein Ladder

The comb was taken out. The glass plates were taken out of the casting frame and set in the cell buffer dam. The running buffer was poured into the inner chamber until the buffer surface reaches the required level in the outer chamber. The samples were then mixed with the sample

buffer. The protein was loaded into the first lane. The prepared solution with the lyophilized garlic extract was loaded into the wells. The top was coved and the electrodes were connected. 65 V voltage was given and the setup was run. The SDS Page was stopped when the down most 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 de-staining solution (10% methanol/7% Glacial Acetic Acid) and agitated on an orbital shaker until resolved blue bands and a clear background appear.

3.2.6 Sub culturing of HaCaT, MG63 and HeLa cells:

Biosafety cabinet was wiped well with 70% isopropanol and left under UV light for 30 minutes and the blower speed was set at 5. Media (DMEM+10% decomplemented FBS+2% antibiotic-antimycotic solution), PBS bottle and Trypsin-EDTA were kept inside the hood after proper wiping with 70% ethanol. Confluent T25 flask was taken out from CO_2 incubator (5% CO_2 95% humidity) and kept on the working bench. Used media was removed using a sterile pipette aid and 2 ml of PBS was added for proper washing. PBS was taken out using the same sterile pipette aid and 2 ml of Trypsin-EDTA was added to remove the surface adherent cells. The flask was then kept into the CO_2 incubator for 2-3 minutes. The flask was then taken out and tapped using a pair of scissors so that all the cells come out in the suspension. 2 ml of fresh media was added and mixed well. Whole cell suspension was taken out in a 15 ml falcon tube and centrifuged at 1000 rpm for 5 minutes. The supernatant was discarded and the cell pellet

was resuspended in 1 ml fresh media. 100µl of the cell suspension was taken out in an eppendorf tube of 1ml and the cells were counted using haemocytometer (Trypan blue dye exclusion test)[27].

3.2.7 Cell counting

After sub-culturing, 10 µl of the harvested cells were taken and counted manually using haemocytometer. Prior to cell loading in the haemocytometer, the cell suspension was diluted with Trypan Blue dye in 1:1 ratio[28]. Thereafter, the cell counting was carried out under phase contrast microscope (Primo Vert, Zeiss). The average cell count was used to estimate the cell concentration using the formula:

Cell concentration (cells/ml) = 4 x average number of cells x 10^4

Specifications of haemocytometer:

- Length = 0.5 mm
- Breadth = 0.5 mm
- Depth = 0.01 mm
- Total Volume = 2.5 x 10^{-3} mm^3

3.2.8 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. 1 ml from this sample was taken and mixed with 9 ml distilled water to obtain 100µg/ml concentration of the sample. 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 prepared[29].

3.2.9 Seeding of HaCaT, MG63 and HeLa cells:

10^4 cells were seeded into each well of a 96 well plate. 200 µl of DMEM media was added to each well. The cells were cultured for one day in an incubator at 37˚C and 5% CO_2. Different sample concentration were added to the wells in triplicates. Wells without any sample were taken as control.

3.2.10 Effect of various cell concentrations on cell adhesion

Sub culturing was done using the earlier mentioned procedure. 3.6 x 10^4 cells were seeded into each well of a 96 well plate. 200 µl of DMEM media was added to each well. The cells were cultured for one day in an incubator at 37˚C and 5% CO_2. Different sample concentration were added to the wells in triplicates. Wells without any sample were taken as control. After certain time intervals, 10µl of the cell suspension was taken and observed under phase contrast microscope. All the analysis was done in triplicates to avoid any error[30].

3.2.11 Effect of garlic extract on the migration of HaCaT and MG63 cells:

Subculturing was done using the earlier mentioned procedure. In a 12 well plate, $2x10^5$ cells were seeded into each well. The cells were allowed to reach confluence and form a sheet. After the cell sheet formation, using a sterile pipette, a scratch was made in each well. The scratch was observed under a phase contrast microscope and images were taken. A location was fixed

to be observed for the next 2-3 days. Samples of different concentrations were added to the wells. After every 24 hours, the scratch was monitored[31].

3.2.12 MTT Assay of seeded HaCaT, MG63 and HeLa cells:

After a day of cell seeding, 20 µl of the MTT reagent was added to each well. Cells were incubated for three hours with the reagent in an incubator at 37˚C and 5% CO_2. The media was removed then and 200 µl DMSO was added to each well to dissolve the purple formazan crystals formed after the incubation. The optical density was measured at 595 nm[32].

CHAPTER 4

RESULTS AND DISCUSSIONS

4.1 UV-Visible Characterization

Sample	Peak Value Wavelength (nm)	Absorbance
Water		
90% Ethanol		
Acetone (Absolute)		
0.1 N NaOH		
0.1 N HCl		

Table 2: UV characterization of garlic scales extracts in various solvents

Samples in water and NaOH give the highest absorbance values indicating maximum phytochemicals extraction in these two solvents. Different peak values show different absorbance wavelengths of various phytochemicals. Peak values from 270-280 nm suggest protein extraction. Peak values in the visible range suggest presence of pigments.

4.2 FTIR Characterization

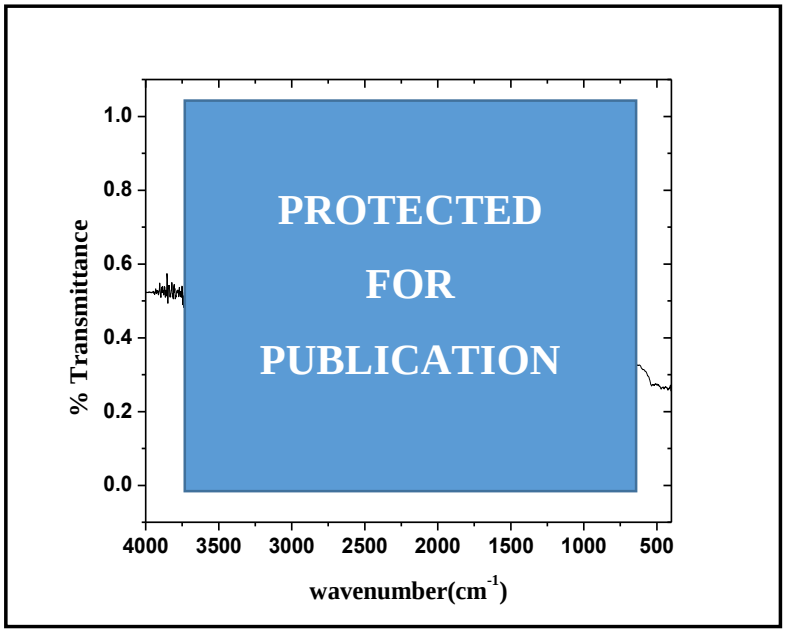


Fig. 1 : FTIR graph for garlic water extract

Wavenumber (cm^{-1})	Functional Group
PROTECTED FOR PUBLICATION	

Table 3: Functional groups corresponding to various wavelengths

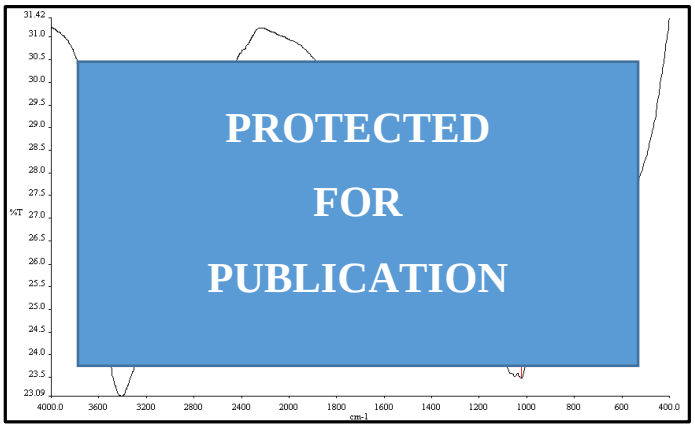


Fig. 2: FTIR graph for lyophilised water extract

The FTIR peaks show presence of thiols, suggesting traces of allicin which may be derived from the bulk garlic. Among other groups, O-H and C=C are present indicating aqueous content and unsaturation.

4.3 Antimicrobial assay

Fig. 3: Antimicrobial assay for 1mg/ml

Fig. 4: Antimicrobial assay for 100µg/ml

Fig. 5: Antimicrobial assay for 10µg/ml

Zone of inhibition was not formed around the well containing the sample at any concentration suggesting either absence of antimicrobial property in the scales or low concentration of the sample.

4.4 SDS PAGE

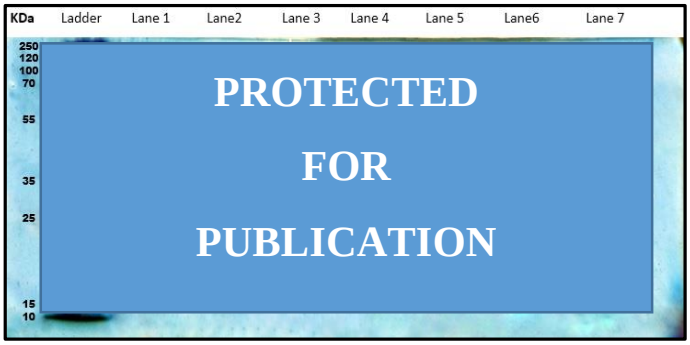


Fig. 6: SDS PAGE

The figure above shows SDS PAGE of lyophilised aqueous garlic extract. As observed from the UV – Visible characterization, the peak values suggested the presence of proteins in the garlic skin extract. From the SDS PAGE experiment, it was confirmed that proteins are present in the sample with a molecular weight of approximately 25 kDa. The proteins act as cell growth modulators. Experiments were conducted to find out whether the extracted proteins have positive or negative effects on the cell growth and proliferation.

4.5 Cell counting using Haemocytometer

Fig. 7: Cell counting

Cell concentration (cells/ml) = 4 x average number of cells x 10^4

Average number of cells = 45

Cell concentration (cells/ml) = 4 x 45 x 10^4 = 1.8 x 10^5

4.6 Cell Adhesion

Table 4: Number of adherent cells at different time intervals

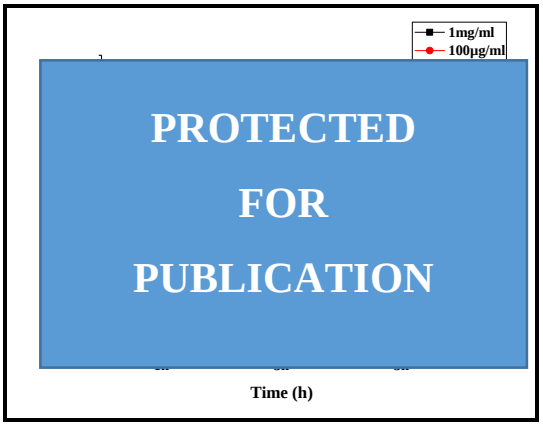


Fig. 8: Percentage cell adhesion vs time

The table gives the number of adherent cells at various time intervals. In the first hour, 1mg/ml concentration reduced the cell adhesion but at the end of 5 hours, the adhesion rate was compensated and the number of non-adherent cells decreased considerably. 100µg/ml sample concentration didn't show any significant effect on the adhesion. 10µg/ml concentration enhances the adhesion and at the end of 5 hours, the number of non-adherent cells were almost negligible.

4.7 Migration

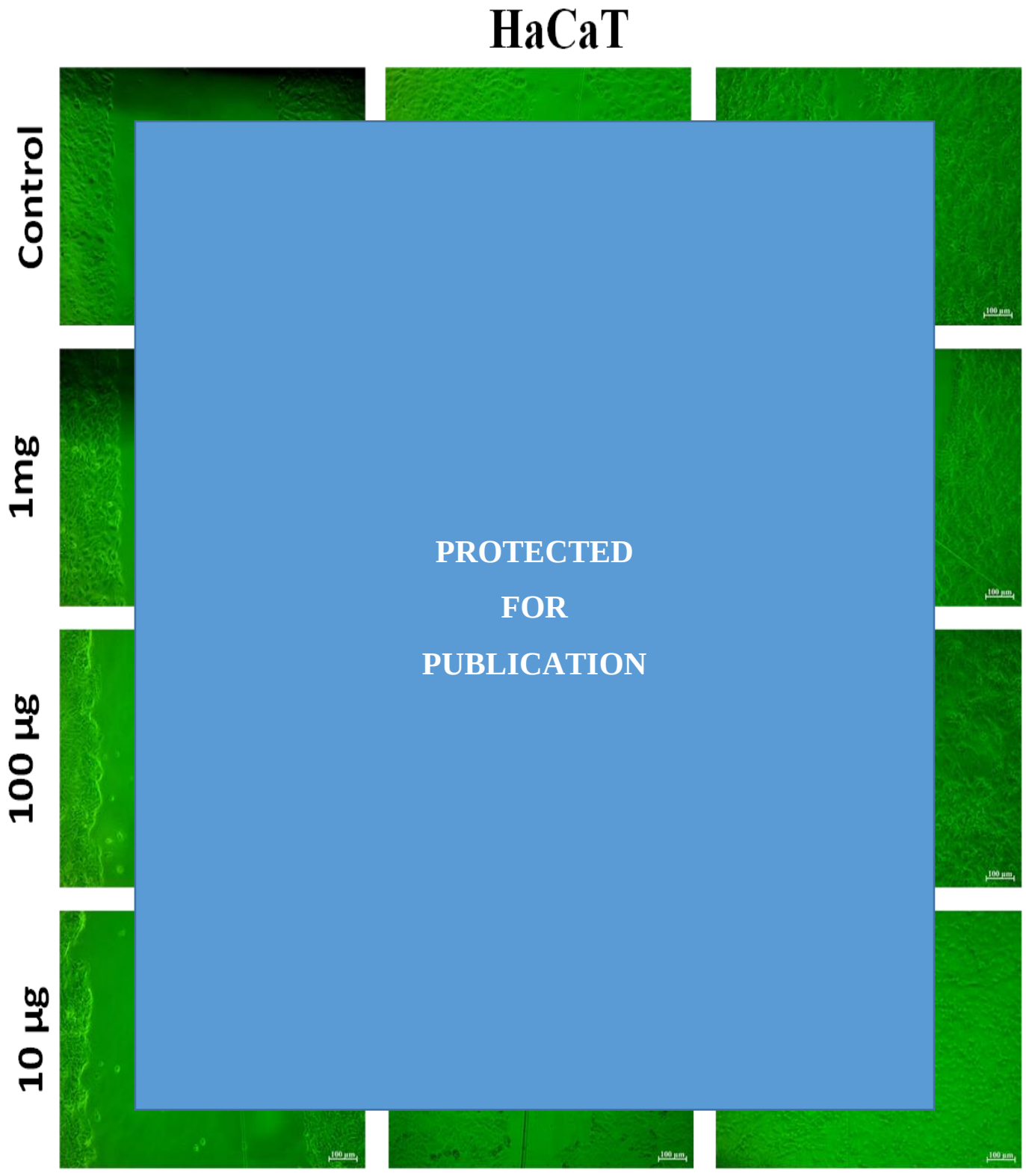


Fig. 9: Migration of HaCaT cells at different concentrations

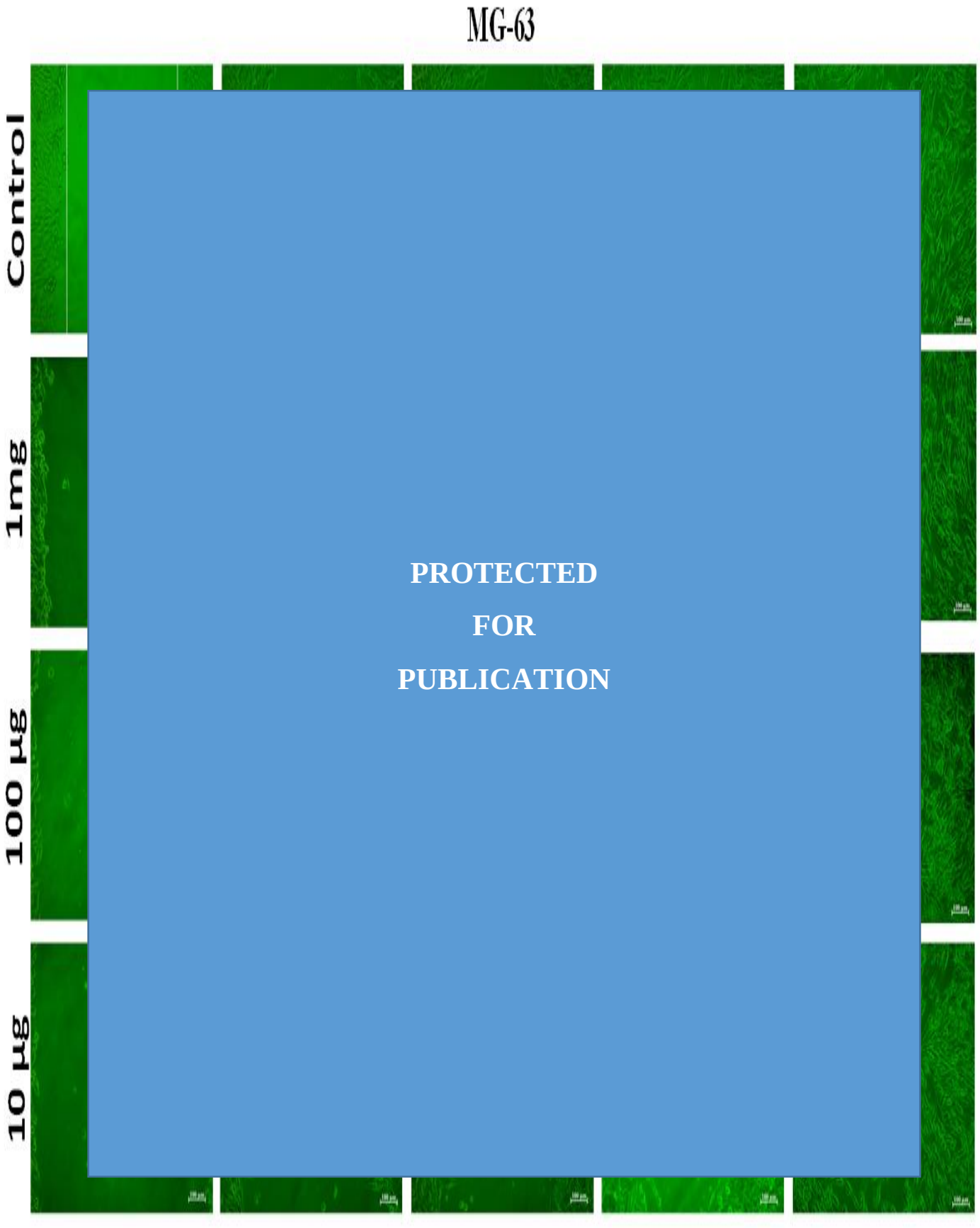


Fig. 10: Migration of MG63 cells at different concentrations

HaCaT	Day 1-Day 0 (µm/day)	Day 2- Day1 (µm/day)
Control		
1mg/ml		
100µg/ml		
10µg/ml		

Table 5: Migration of HaCaT cells at different concentrations

MG63	Day 1-0 (µm/day)	Day 2-1 (µm/day)	Day 3-2 (µm/day)	Day 4-3 (µm/day)
Control				
1mg/ml				
100µg/ml				
10µg/ml				

Table 6: Migration of MG63 cells at different concentrations

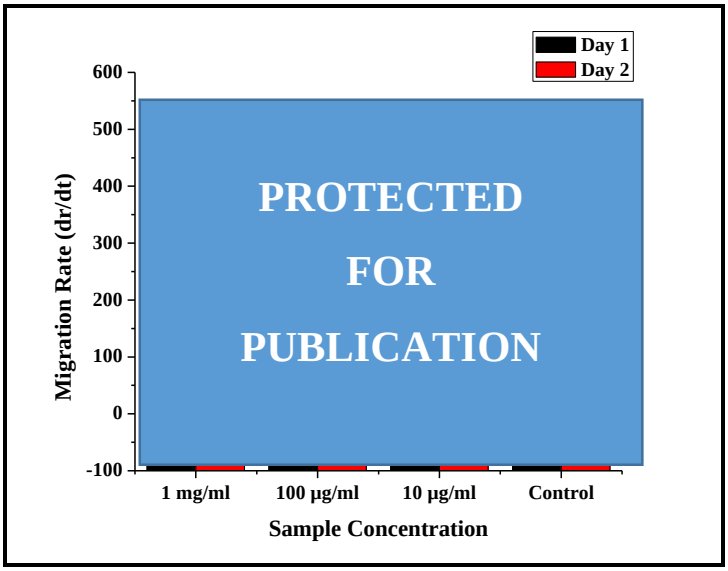


Fig. 11: Graph showing migration of HaCaT cells at different concentrations

The graph represents migration of HaCaT cells with different sample concentrations. The rate of migration is highest at 10µg/ml concentration. The cells tend to die and float away at 1mg/ml concentration thus increasing the distance from the scratch. For every sample concentration except 1mg/ml, migration is more on the first day. For 100µg/ml concentration, the migration rate decreases considerably on the second day.

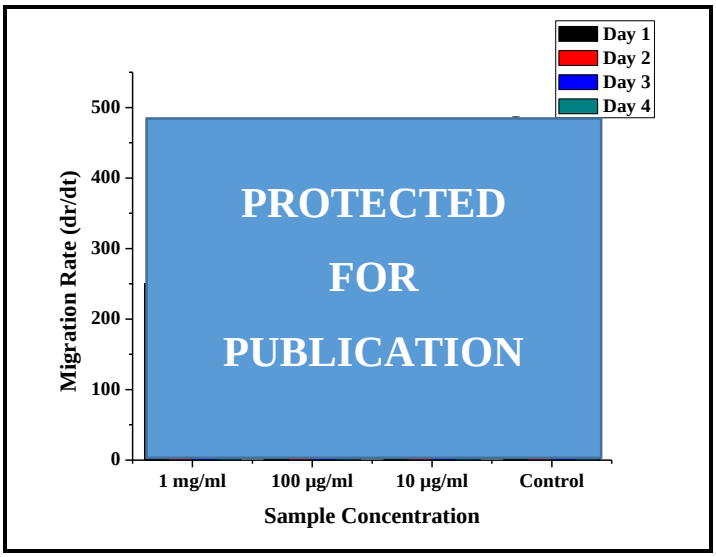


Fig. 12: Graph showing migration of MG63 cells at different concentrations

The graph represents migration of MG63 cells with different sample concentrations. On the first day, migration of cells without the samples is more than the cells containing the samples. For 1mg/ml and 10µg/ml, the migration increases on the second day. On the third day, migration of cells with sample concentration 100µg/ml increases considerably and the rate remains the same even on the fourth day. For 10µg/ml, the migration reaches the maximum on the fourth day. Except for 100µg/ml concentration, the scratch is completely closed on the fourth day.

4.7 Cell Viability by MTT Assay

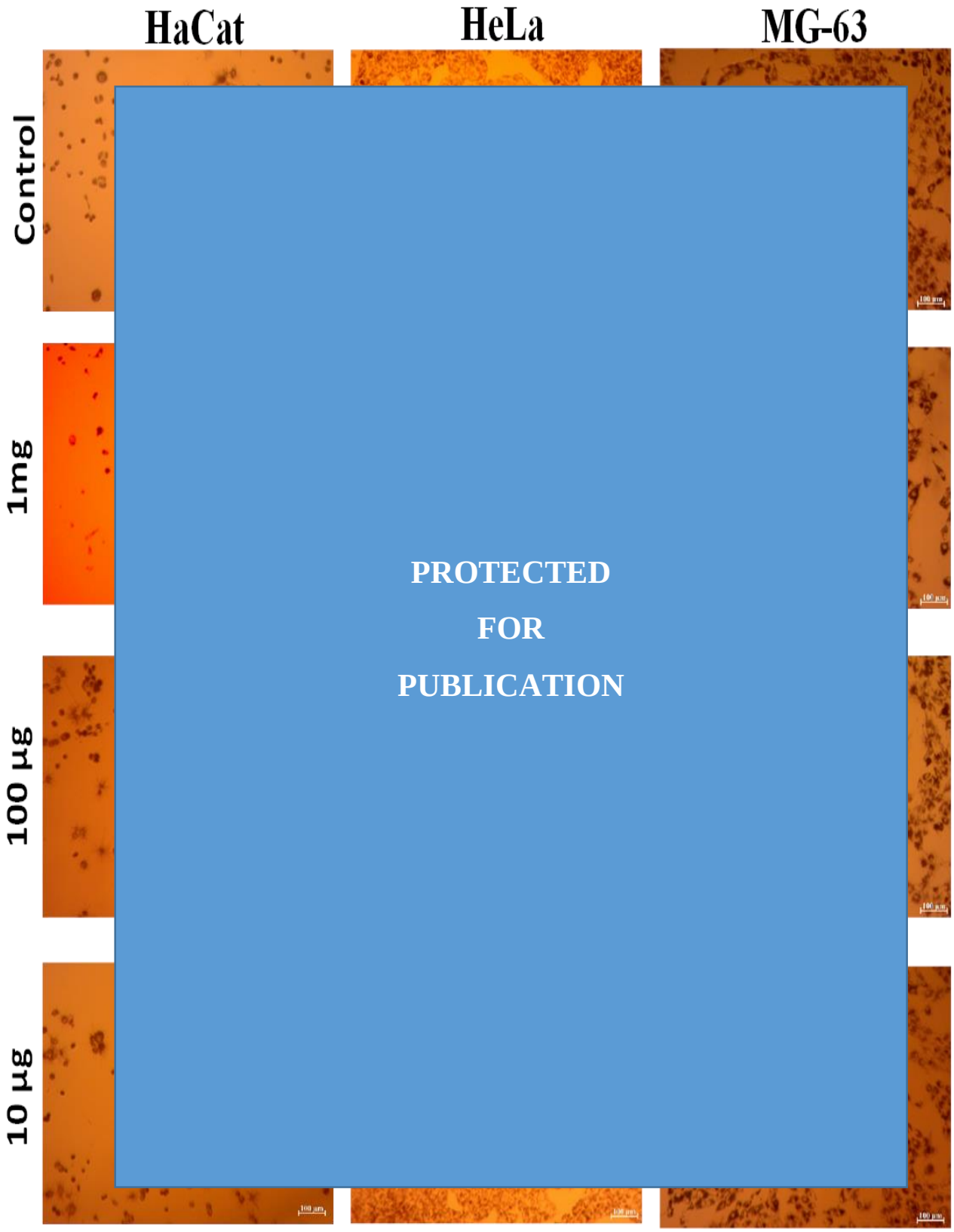


Fig. 13: MTT Assay for various sample concentrations in three cell lines

Concentraion	HeLa	MG63	HaCaT
1mg/ml			
100µg/ml			
10µg/ml			
Control			

Table 7: Absorbance values for MTT Assay

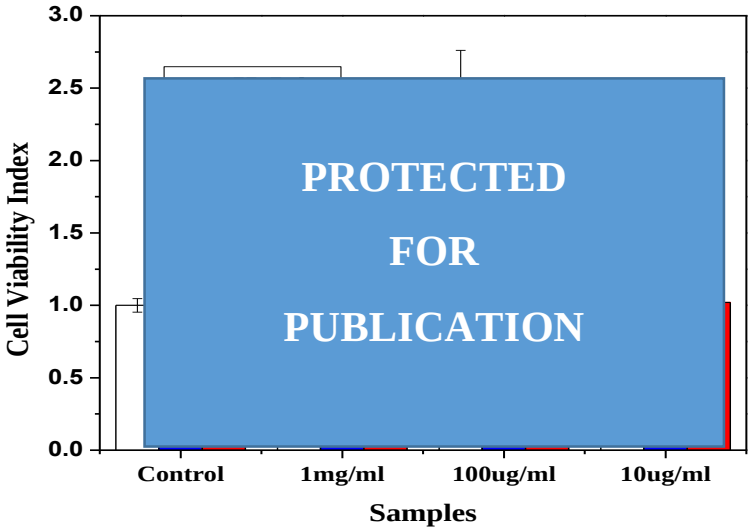


Fig. 14: Graph depicting migration rate for various concentrations

The cell viabilities of different cell lines were compared with the addition of various sample concentrations. It was observed that 100µg/ml concentration when added to the HeLa cells gives the highest viability index inferring an increase in the proliferation of HeLa cell line. 1mg/ml sample concentration is detrimental to the viability of HaCaT and MG63 cells. At every sample concentration, HeLa cells give the maximum proliferation.

CHAPTER 5

CONCLUSION

CONCLUSION

Active component obtained from garlic scales were chemically characterized and their effects on cell physiology was observed for three different cell lines. UV-Visible characterization of the scales suggested presence of proteins which was confirmed later using SDS PAGE. Using Fourier Transform Infra-Red spectroscopy, thiol group was identified which suggested traces of allicin or a related protein in the scales. The cellular adhesion, migration and proliferation under the influence of garlic extract clearly showed that the compounds from the garlic scales can be used as a biological response modifier. The studies are at a preliminary stage and for better understanding, more detail chemical characterization and subsequent in vivo studies should be performed.

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