International Journal of Herbal Medicine
Available online at www.florajournal.com

E-ISSN: 2321-2187
P-ISSN: 2394-0514
www.florajournal.com
IJHM 2021; 9(1): 93-99
Received: 02-12-2020
Accepted: 06-10-2021

Gayatri G Gore
Cancer Biology Department, Genesis Labs, Godavari Biorefineries Ltd, Maharashtra, India

Smera Satish
Cancer Biology Department, Genesis Labs, Godavari Biorefineries Ltd, Maharashtra, India

Akshay Ganpule
Cancer Biology Department, Genesis Labs, Godavari Biorefineries Ltd, Maharashtra, India

Sangeeta Srivastava
Cancer Biology Department, Genesis Labs, Godavari Biorefineries Ltd, Maharashtra, India

Maithili Athavale
Cancer Biology Department, Genesis Labs, Godavari Biorefineries Ltd, Maharashtra, India

Corresponding Author:
Maithili Athavale
Cancer Biology Department, Genesis Labs, Godavari Biorefineries Ltd, Maharashtra, India

Garlic (*Allium sativum*) exhibits anticancer and anticancer stem cell activity on Breast, Prostate, Colon, Hepatic and Cervical cancer cell lines

Gayatri G Gore, Smera Satish, Akshay Ganpule, Sangeeta Srivastava and Maithili Athavale

Abstract
Garlic (*Allium sativum*) is well known for its anticancer properties, but till date a detailed study on anticancer and anticancer stem cell activity on various types of cancer cell lines has not been reported. We have described a comprehensive study of three garlic extracts on various types of cancer cell lines. One of the extracts has exhibited highest activity in MTT assay. It has also shown cancer stem cell inhibition in all the cell lines tested at average concentration of 0.305 mg/ml. In case of wound healing assay it has again exhibited maximum inhibition at scratch for MDAMB231 (88% ±1.4), PC3 (94% ±4.7), Hep3B (98% ±2.16) & HeLa (98% ±0.85) at 48 hours. All extracts were analyzed by HPLC for presence of active components Allin and Allicin of Garlic. In short, this potent Garlic extract with anticancer and anticancer stem cell activity which can be used as a natural supplement during chemotherapy.

Keywords: Garlic extracts (*Allium sativum*), wound healing/scratch assay, CSC Sphere inhibition, MTT, Normal Peripheral Blood Lymphocytes

1. Introduction
Garlic (*Allium sativum*) has long been consumed commonly not only as food by humans but also as a traditional medicine [1, 2]. A detailed study of therapeutic uses and pharmacological properties of Garlic (*Allium sativum*) has also been reported [3]. Cancer comprises of a heterogeneous cell population with a small fraction of tumour initiating cells called Cancer Stem Cells (CSCs) [4]. CSCs have the ability of the self renewal, unlimited propagation and multipotent differentiation. Due to this and other characteristics, CSCs are inherently resistant to conventional cytotoxic agents [5]. Chemotherapeutic drugs end up killing cancer cells sparing CSCs which after a while can give rise to a full blown tumour with high metastatic potential. These drugs mainly involve the use of synthetic compounds [6]. Thus the need of the hour is to have a natural agent which can possess both anticancer and anticancer stem cell activity and additionally should have no or minimal effect on normal cell population.
Anticancer property of garlic extract has been reported and there are reports of its activity on a few cancer cell lines [7]. Till date there is no reported comprehensive study of garlic extract on different types of cell lines. Although there are a few reports indicating anticancer stem cell activity of garlic on Breast [8] and Glioblastoma cell line [9], there is a lack of detailed report on its anticancer stem cell activity on various types of cell lines.
Our paper describes the *in-vitro* testing of three different types of garlic extracts G1, G2 and G3 for anticancer and anticancer stem cell activity on various cancer cell lines like; Breast (MDAMB231, MDAMB468 and T47D), Prostate (PC3 and DU145), COLON (COLO205, COLO 320DM and HCT-15), Cervical (SiHa and HeLa) and Hepatic (Hep3B). We also report our study on toxicity of these extracts on normal peripheral blood lymphocytes (PBLs).

2. Materials and Methods
2.1 Materials
The fresh garlic (Bhima Purple) was collected from farm, Shirdi (Maharashtra, India) in November 2017. Our laboratory has received permission from National Biodiversity Authority (NBA) to screen Garlic (*Allium sativum*) for anticancer and anticancer stem cell properties.

2.2 Methods
Garlic cloves were peeled and blended with solvents like; 40% ethanol and HPLC (High Performance Liquid Chromatography) grade water to get three types of garlic extracts G1, G2

and G3 respectively. Briefly, 70g of cleaned garlic cloves were crushed in 50 ml of 40% ethanol using a bench top blender to obtain a fine paste. This crude garlic ethanol mixture was transferred to a glass jar with an air-tight lid with additional 40 ml of 40% ethanol and stored in dark at 4ºC for 5 days. The supernatant was then squeezed through a muslin cloth. The dry mass was discarded and supernatant was further centrifuged at 5000 r.p.m. for 10 minutes at 4ºC and labeled as G1 [10]; while preparing G2, 20g of cleaned garlic cloves were grounded in 50ml of HPLC grade water. This crude garlic paste was transferred to a glass jar with a air-tight lid and heated in water bath at 60ºC for 6 hours and was kept on shaker overnight at room temperature. The liquid was then squeezed through a muslin cloth and centrifuged at 5000 r.p.m. for 10 minutes. The supernatant collected was passed through 0.2µ filter and labeled as G2 [11]. We have concentrated 20 ml of G1 by evaporating it under sterile condition for 24 hours to obtain a viscous liquid residue and named as G3. Further G1, G2 and G3 were subjected to microbial sterility testing and were found to be sterile.

These extracts were tested for anticancer property by MTT assay and anticancer stem cell property by CSC sphere formation inhibition assay and Wound/Scratch Healing Assay and toxicity by testing activity on PBLs.

2.2.1 MTT assay

Cell lines used in a study were procured from NCCS Pune, India. Anticancer activity of G1, G2 and G3 was tested by MTT assay [12]. Briefly, Cancer cells were cultured in 96-well tissue culture adherent plates with respective medium and a predetermined plating efficiency as mentioned in Table.1, the cell culture media were procured from HiMedia (Mumbai, India). The media were supplemented with 10% Fetal Bovine Serum (HiMedia, Cat No. RM1112) and Antibiotic – Penicillin and Streptomycin (HiMedia Cat No. A002). In short 200 uL of these cells with appropriate platting efficiencies were seeded in each well of 96-well plates and incubated at 37ºC for 24 hours in 5% CO_2 to obtain a log phase culture. The monolayered cells were then exposed to various dilutions of the extracts. After adding the extracts to the cells they were further incubated at 37ºC for 48 hours 5% CO_2 and the assay was terminated by centrifuging the plates at 3000 r.p.m. for 3 minutes. The supernatant was then removed followed by addition of 100 uL of 0.5mg/ml of MTT [3-(4,5-Dimethylthiozol-zyl)2,5-Diphenyl Tetrazolium Bromide solution (Sigma, Cat No. M2128). The plates were incubated for 4 hours at 37ºC in 5% CO_2. During this incubation period the MTT gets metabolically reduced by viable cells to yield a blue insoluble formazan product. After addition of 200 uL of DMSO (SDFCL, Cat No. 38216) formazan product gets solubilized and is measured at 570nm spectrophotometrically.

Controls such as Growth Control (GC) comprising Cells + Medium, Growth control with DMSO (GCD), Growth control with HPLC water (GCH), Growth control with 40% Ethanol (GCE) and Medium Control (MC) were set up for each set of cell lines. Drug Positive Controls (DPC) with concentration of 0.5M Cisplatin were also set up and it has exhibited 100% cell death in all the experiments. Each assay was performed as 6 data points for each concentration of the garlic extract. The mean of cell viability values were compared to respective Growth Controls to calculate the% viability for each dilution. A X-Y graph of log% viabilities was then plotted against respective log drug concentrations. Drug concentration inhibiting the growth of 50% cell population (IC50) was then calculated for each extract by regression analysis using Microsoft Excel 2007.

Table 1: The media and plating efficiencies used in MTT assay to determine potency of G1, G2 and G3:

Cell Lines Type	Cell Line	Complete Medium (1X)	Plating Efficiency (cells per well) x 10^5
Breast	MDAMB231	Dulbecco's Minimal Essential Medium	0.1
	MDAMB468	Dulbecco's Minimal Essential Medium	0.15
	T47D	RPMI 1640 with 1M HEPES	0.2
Prostate	PC3	RPMI 1640	0.1
	DU145	Dulbecco's Minimal Essential Medium	0.05
Colon	COLO205	RPMI 1640	0.15
	COLO320DM	RPMI 1640	0.2
	HCT-15	RPMI 1640	0.15
Cervical	HeLa	RPMI 1640	0.05
	SiHa	Minimal Essential Medium	0.1
Hepatic	Hep3B	Minimal Essential Medium	0.1
Normal Peripheral Blood Cells	Lymphocytes	RPMI 1640	7.5

2.2.2 CSC sphere inhibition assay

Sphere assay measures the ability of CSCs to form spheres in specially designed serum free medium [13]. We have used this assay to measure the killing efficiency of garlic extracts (G1, G2 & G3) on CSC population of Breast, Prostate, Colon, Cervical & Hepatic cell lines. We have modified the existing protocol and standardized this in our laboratory [14]. Briefly, Log phase cell culture of all cell lines were trypsinized and made into single cell suspension by passing through cell strainers of 100 µ (Tarson, Cat No. 352360) and 40 µ (Tarson, Cat No. 352340) respectively. The cells were diluted at the concentration of 2000 cells/ 100 µL/ well and suspended in either Mammosphere (Lonza, Cat No. CC3151- for Breast Cell Lines), Prostosphere (Lonza, Cat No. CC3165- for Prostate cell lines) and DMEM F12 (Himedia, Cat No. AL187A, for Cervical, Hepatic and Colon cell lines). These media were further supplemented with FGF (Sigma, Cat N0. PFG0266), EGF (Sigma, Cat No. E9644), B27 Supplement (Gibco, Cat No. 17504044), Antibiotic Antimycotic solution (Himedia, Cat N0. A002) and Heparin (Samarth, 25000 IU). 100 µL of this complete medium was then added to each well of 96-well suspension plates and incubated at 37ºC for 24 hours, 5% CO_2. Appropriate concentrations of garlic extracts G1, G2 & G3 were added into respective wells along with 100 µL of complete medium. Plates were incubated at 37ºC for 72 hours, 5% CO_2. After incubation second feeding of media (50 µL) and respective concentration of extracts addition (2.5 µL) was performed. Plates were again incubated at 37ºC for 72 hours, 5% CO_2. After incubation, the last feeding of media (50 µL) and respective concentration of extracts addition (3

μL) was performed. Plates were incubated at 37ºC for 24 hours, 5% CO_2. Thus the Primary spheres for each cell lines, formed after 9 days, were counted microscopically (10X). A comparative graph of Concentrations (mg/ml) inhibiting 50% of spheres formation was plotted against each cell line for all the extracts (Fig.2).

2.2.3 Wound Healing/ Scratch Assay

From each group of the cell lines, the cell lines forming confluent monolayers were selected for Wound Healing/ Scratch Assay [15]. The cell lines selected were MDAMB231, PC3, Hep3B and HeLa.

As none of the colon cancer cell lines formed a confluent monolayer we did not select them for this assay. For the experiment, $6x10^5$ cells of each cell line were seeded onto 6-well tissue culture plates for 48 hours to allow the formation of confluent monolayer.

A single scratch at 0 hours was created using sterile 200 μL micropipette tip. Cells were washed thrice with DPBS (Himedia, Cat No. PL1006) to remove cell debris supplemented with assay medium. The width of the scratch was immediately recorded in mm using software IS-Capture camera measure. Briefly, IC10 concentrations of each the garlic extracts G1, G2 & G3 obtained from MTT assay (Table.1) were selected for experimental purpose. Dilutions of the extracts were prepared in 40% ethanol for G1, G3 and in HPLC grade water for G2.

Suitable controls GC, GCH & GCE were also set up for each experiment. The experimental plates were incubated at 37ºC at 5% CO_2 and the width of the scratch was recorded at 24 and 48 hours respectively for each cell line. A comparative graph of% growth versus Garlic extracts for selected group of cell lines was plotted (Fig.3).

2.2.4 Normal Peripheral Blood Lymphocyte (PBLs) Assay

In order to check toxicity of garlic extracts on normal PBLs, we have standardized following procedure in our laboratory. Human lymphocytes were isolated from peripheral blood, by overlaying the diluted defibrinated blood on a solution of sodium diatrizoate and polysucrose (HiSep, Cat No. LSM 1077) and centrifuged at 2300 r.p.m. for 30 minutes at 4^0C. The lymphocyte layer (the buffy coat) obtained was carefully removed in a new collection tube, washed twice by DPBS and centrifuged at 1900 r.p.m. for 10 minutes at 4ºC. The supernatant was discarded and pellet was resuspended in DPBS. After confirming the viability and purity (>95%), the cells were diluted to concentration of $0.75x10^6$ cells per ml and were taken for MTT assay as described earlier.

2.2.5 HPLC Analysis

Garlic (*Allium sativum*) has been used in world cuisines as well as in herbal medicine for thousands of years. It is known that Allicin is the main biologically active component of freshly crushed garlic-*Allium sativum*. HPLC was performed on the three extracts G1, G2 and G3. Briefly, HPLC system consisted of Isocratic mode of Methanol: Water (60:40) and C18 column.

UV detector was set at 240nm for all operations. Chromatogram (Fig.3) of G1, G2 and G3 indicate peak at 3.7 mins which corresponds to standard Allicin peak analyzed under similar conditions [16].

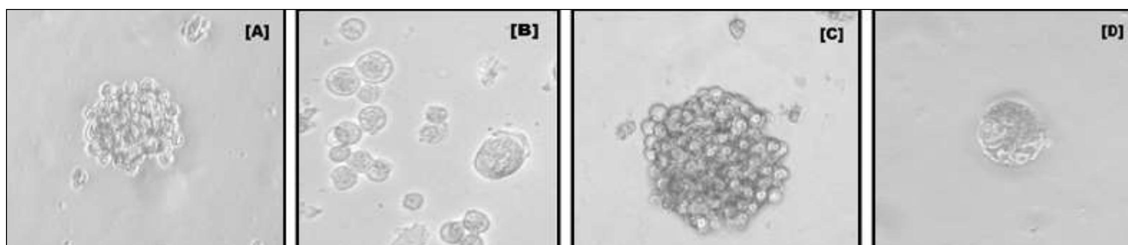
3. Results and Discussion

3.1 The IC50 value in mg/ml of three garlic extracts G1, G2 and G3 when tested across various cancer cell lines and Human Lymphocytes

Table 2: Represents the IC50 values (mg/ml) obtained by the activity of G1, G2 and G3 on Breast, Prostate, Colon, Cervical, Hepatic and even on normal peripheral blood lymphocytes in MTT assay. A close observation of Table.2 reveals that amongst all the garlic extracts G3 is exhibiting very high potency on all the types of cancer cell lines. Further the activity of G1, G2 and G3 on normal peripheral blood lymphocytes revealed that G2 is non toxic on Lymphocytes whereas G2 and G3 exhibit some toxicity. Though G3 is exhibiting activity on lymphocytes, it's activity is much lower compaired to it's activity on cancer cells ($p<0.05$).

Cell lines	G1	G2	G3
	IC50 values in mg/ml (n=6)		
MDAMB231	5.748 (± 0.002)	No activity	0.849 (± 0.004)
MDAMB468	4.364 (±0.0028)	3.141 (± 0.0021)	0.616 (± 0.0007)
T47D	No activity	No activity	0.833 (± 0.0035)
PC3	3.333 (±0.004)	2.71 (± 0.0035)	0.617 (± 0.0028)
DU145	3.674 (± 0.0035)	2.458 (± 0.035)	0.855 (± 0.0014)
COLO205	No activity	No activity	0.483 (± 0.021)
COLO320DM	No activity	No activity	0.934 (± 0.028)
HCT-15	3.746 (± 0.0021)	3.334 (± 0.0014)	0.678 (± 0.0042)
HeLa	4.165 (± 0.0042)	2.680 (± 0.0035)	0.721 (± 0.0028)
SiHa	3.916 (± 0.0014)	3.443 (± 0.007)	0.703 (± 0.002)
Hep3B	3.301 (± 0.0021)	3.079 (± 0.0028)	0.624 (± 0.0014)
PBLs	5.903 (± 1.004)	No activity	1.192 (± 0.12)

3.2 Tumor spheres



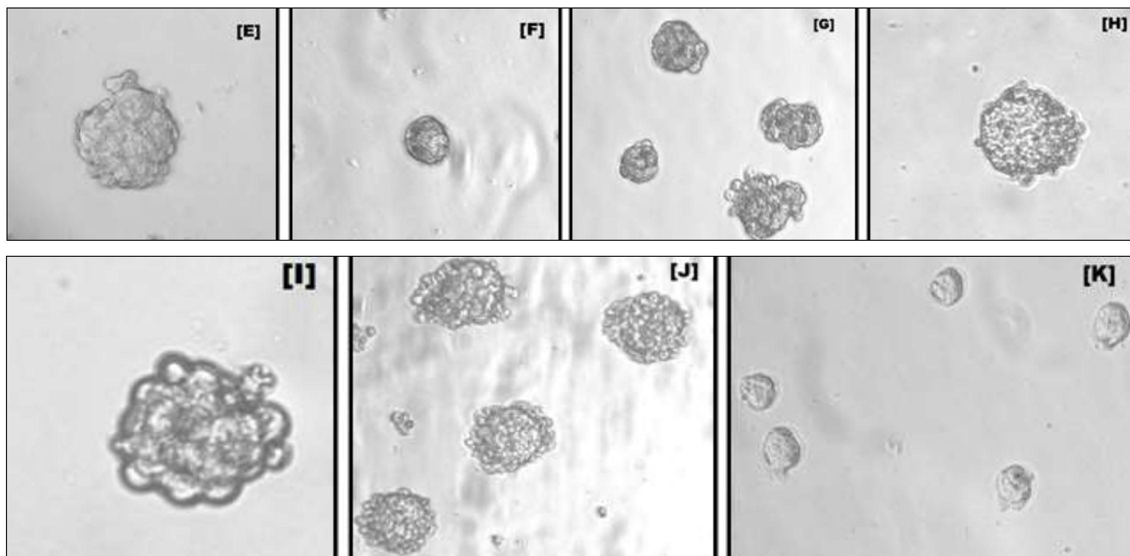


Fig 1: Represents the photographs of spheres formed in CSC sphere formation inhibition assay with respect to GCD control. [A] Mammosphere of MDAMB231, [B] Mammosphere of MDAMB468, [C] Mammosphere of T47D, [D] Prostosphere of PC3, [E] Prostosphere of DU145, [F] Sphere of COLO205, [G] Sphere of COLO320DM, [H] Sphere of HCT-15, [I] Sphere of HeLa, [J] Sphere of SiHa, [K] Sphere of Hep3B.

Fig.1 indicates the tumorospheres of Breast, Prostate, Colon, Cervical and Hepatic cell lines respectively. For cancer stem cell sphere formation inhibition assay, the concentration range selected was from 17.5 to 0.0305 mg/ml. The results of anticancer stem cell activity obtained from sphere assay are depicted in Fig.2. It can be clearly seen that G3 has the highest potential to inhibit cancer stem cells on all the cancer cell lines tested. The concentration at which the 50% spheres inhibited by G3 was found to be 0.305 mg/ml for all cell lines except for COLO320DM and PC3 where the concentration was found to be 0.62 and 1.54 mg/ml respectively. The extracts G1 and G2 have exhibited a much lower anticancer stem cell activity compare to G3.

3.3 The anticancer stem cell activity of three garlic extracts G1, G2, and G3 across various cancer cell lines in CSC sphere inhibition assay

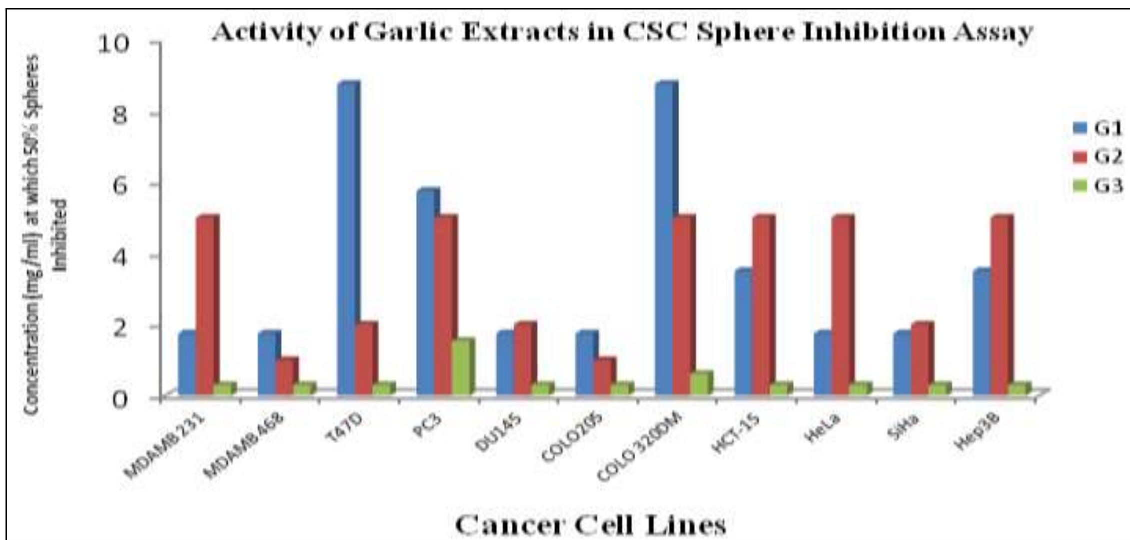


Fig 2: Indicates the comparative anticancer stem cell activity of three garlic extracts G1, G2, and G3 across various cancer cell lines in CSC sphere formation inhibition assay. It can be clearly seen that G3 is exhibiting highest anti-CSC activity on all the cancer cell lines.

3.4 The comparative% Growth in cancer cell lines when exposed to garlic extracts in Wound/Scratch assay

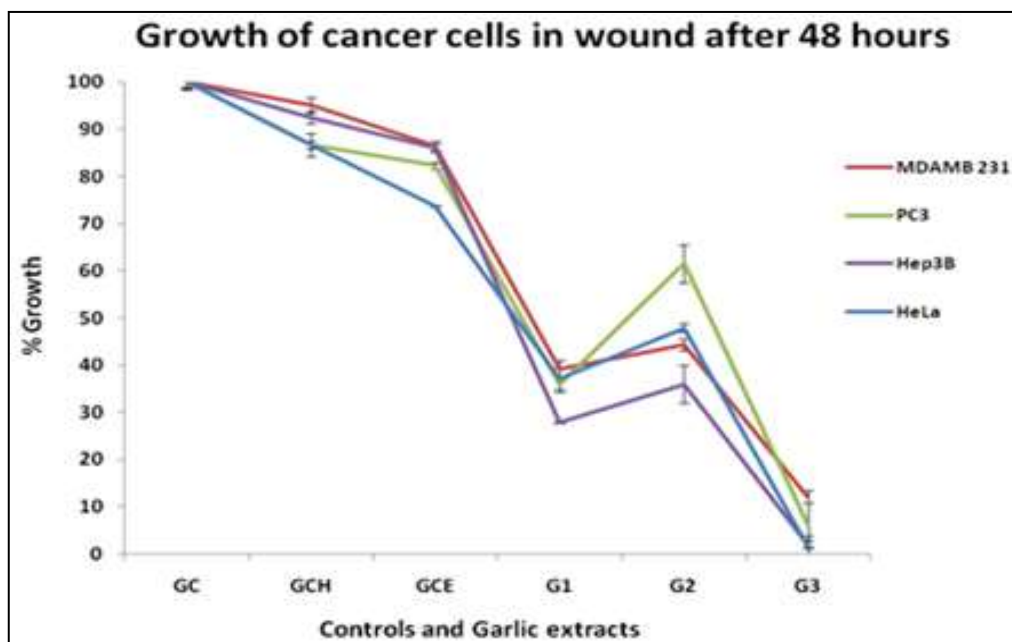


Fig 3: indicates growth of cancer cells in wound / scratch after 48 hours in cell lines MDAMB231, PC3, Hep3B and HeLa in G1, G2 & G3 treated cells. GC- Growth control, GCH- Growth control for HPLC water, GCE- Growth control for Ethanol.

In addition to sphere assay, we have also performed Wound Healing/ Scratch Assay to understand the anticancer stem cell effect. The cell lines, forming a confluent monolayer (MDAMB231, PC3, HeLa and Hep3B) were selected for this assay. Fig.3 clearly indicates that the G3 has the highest potential for% inhibition at scratch for all the cancer cell lines tested suggesting a strong anticancer stem cell effect. It also reveals the results of wound/scratch assay for cell lines MDAMB231, PC3, Hep3B and HeLa when exposed to G1, G2 and G3. It can be clearly seen that G3 exhibits minimum% Growth at the end of 48 hours compared to G1 and G2 indicating maximum effect on all the cancer cell lines.

3.5 Wound Healing / Scratch Assay

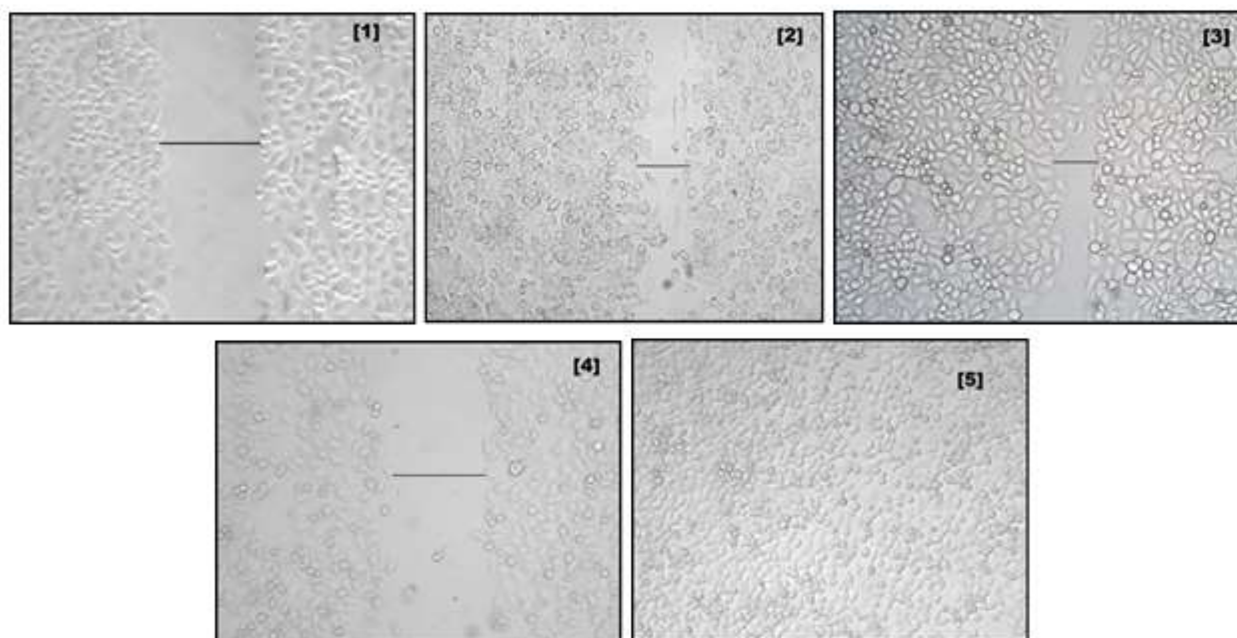


Fig 4: indicates the images of scratch assay with PC3 cells [1] After Scratch – 0 hour, [2] G1 – 48 hours, [3] G2 – 48 hours, [4] G3 – 48 hours, [5] GC – 48 hours.

The width of the scratch was determined by using IS-Capture software in mm. In case of PC3, the width of scratch at 0 hour was 32mm. After 48 hours, the width of scratch observed was G1= 20.54mm, G2= 12.31mm, G3=30.02mm and in GC showed complete healing of the wound. Thus, width of the scratch in G3 remains almost same at the end of 48 hours indicating maximum effect on cancer cells.

3.5 HPLC Analysis Report

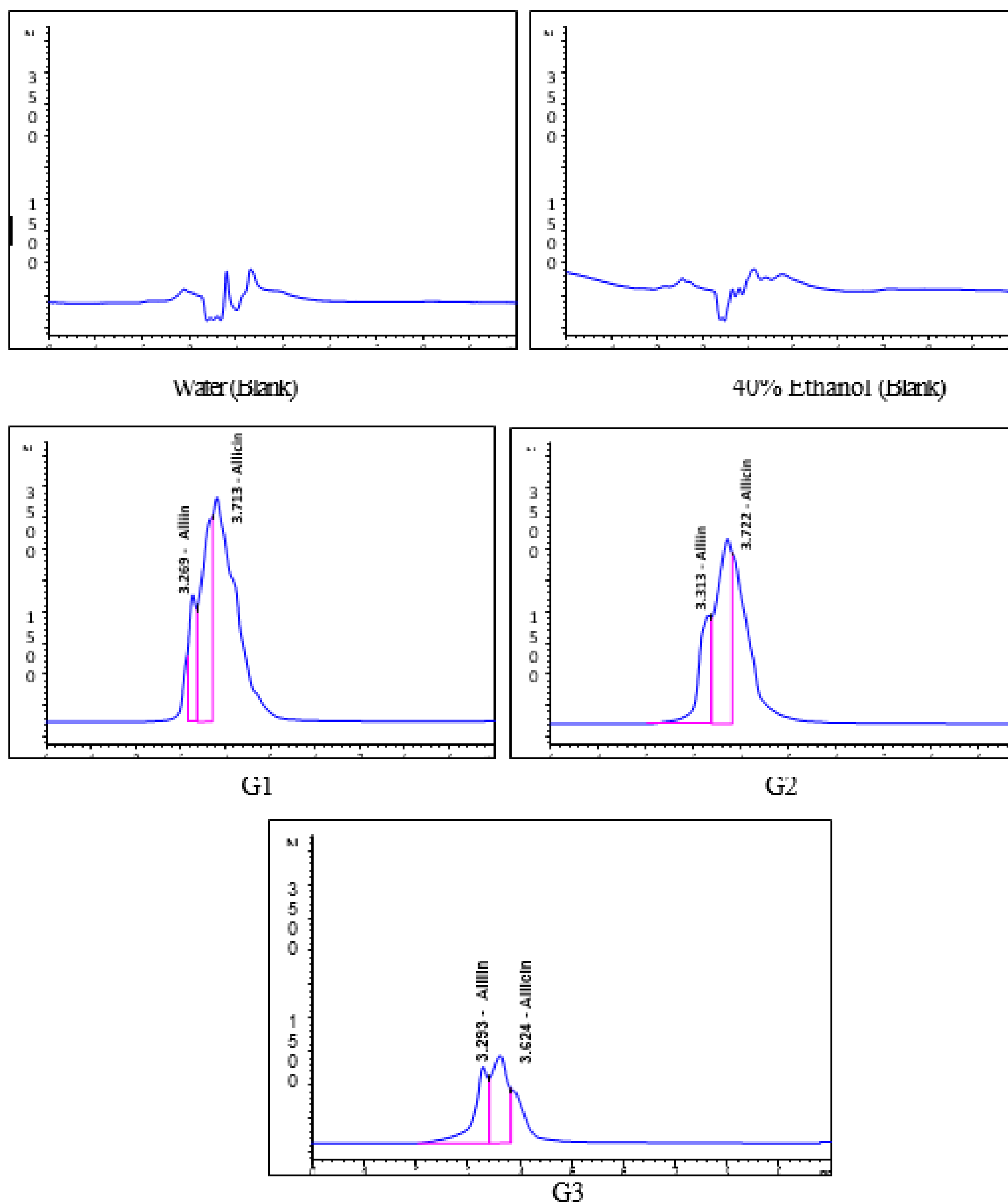


Fig 5: Indicates the HPLC chromatograms of G1, G2 and G3 when analyzed for the presence of Allin and Allicin.

4. Conclusion

Cancer stem cells due to their properties of self renewal, quiescence, differentiation, chemo resistance and ability to recapitulate the parental tumour when transplanted into a host co-ordate with poor clinical outcome in case of cancer patients [17]. Considering the severe side effects posed by current chemotherapeutic drugs, there is an urgent need of a potent natural agent which can aid in the elimination of not only the cancer cells but also the cancer stem cells. Anticancer properties of Garlic (*Allium sativum*) is well described but till date a detailed comprehensive study of anticancer and anticancer stem cell effect of Garlic on various cell lines has not been described. Here we have reported a detailed scientific study of three extracts G1, G2 and G3 Prepared from fresh Garlic cloves. MTT, Sphere Assay and Wound Healing Assay clearly indicate the potencies of the extracts. Allicin, the main organic allyl Sulphur component in Garlic is known to exhibit antitumor activity. We have also analyzed the Allicin content of the extract by HPLC and have found the presence of Allicin in all the three extracts (Fig.5). G3 has exhibited a very high anticancer and anticancer stem cell activity on various types of cancer cell lines tested in the study. The cancer treatment generally follows surgery, radiation, chemotherapy or combination of these. Most of the time chemotherapy involves the use of synthetic drugs which possesses high toxicity [18]. These drugs only kill cancer cells and spare cancer stem cells thus resulting in tumour relapse after some time. Further, the results of the study need to be confirmed by In-vivo methods. Garlic extract thus can be used as a natural anticancer stem cell agent along with standard of care chemotherapeutic drug to lower the toxicity and for completely alleviating cancer.

5. Acknowledgement

Authors are grateful to Shri. Samir Somaiya (M.D. and Chairperson of Godavari Biorefineries Ltd.) for financial support and encouragement and Dr. Sendurai Mani (Professor M.D. Anderson) for invaluable guidance. We are thankful to

Mr. D. V. Deshmukh for research support. We are also thankful to Mr. Rajiv Patil and Mr. Bhushan Patil from Analytical Department, Genesis Labs -Mahape for their help in HPLC analysis.

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