Anti-cancer activity of *Zingiber officinale* in HEp-2 Cell line

Madhubala, Department of Biotechnology, Faculty of Engineering & Technology, Rama University, Uttar Pradesh, Kanpur, India. madhu02july@gmail.com

Vivek Shrivastava, Department of Biotechnology, Faculty of Engineering & Technology, Rama University, Uttar Pradesh, Kanpur, India. viveksrivastavabio@gmail.com

Ajay Kumar, Department of Biotechnology, Faculty of Engineering & Technology, Rama University, Uttar Pradesh, Kanpur, India. ajaymtech@gmail.com

Abstract: Ayurveda is a traditionary system of medicine in India. Ayurveda focuses on the plants for the medicinal potential. Ginger is the plant which recognized well in Ayurveda. These are known for its anti cancerous effect. Ginger (*Zingiber officinale*) is the most widely used natural products consumed as a spice and medicine for treating nausea, dysentery, heartburn, flatulence, diarrhea, loss of appetite, infections and bronchitis. Experimental studies showed that ginger and its active components including 6-gingerol and 6-shogaol exert anticancer activities. Active compound of Ginger like 6-gingerol and 6-shogaol are used for the treatment of cancer. Active compound of Ginger 6-shogol have ability to modulate the various signaling molecules. This study is designed to examine the in vitro cytotoxic activities of ethanolic extract prepared from ginger extract on HEp-2 cell line. The cytotoxic effects of the drugs are confirmed by MTT assay and NRU assay and agarose gel electrophoresis is performed for determination of the induction of apoptosis. Among all concentrations 1000µg/ml shows cell growth inhibition. Further, some amount of damaged DNA is also observed by gel documentation in DNA fragmentation assay. The results of the present study suggest that ginger might be useful as a potential anti tumour agent.

***Keywords: MTT Assay, NRU Assay, Cell line, DNA Fragmentation, Anti cancer activity, Cytotoxicity*.**

I. INTRODUCTION

Cancer is likely to remain a major risk to public health for many more years[1]. Despite new advancements in diagnosis, therapy and prevention, cancer still affects millions of people worldwide and is one of the leading of death[2]. Cancer is the unusual growth of cells in our body that can proceed to death. Cancer cells generally invade and destroy normal cell. Gradually more and more cancer research works have been done and yet we do not know what cancer is? It is currently recognized that deregulated cell proliferation and apoptosis direct to the development of cancer. Several agents, both naturally occurring agent and synthetic agents, have been studied for anticancer properties. These naturally occurring agents and synthetic agents often induce tumour cells to undergo apoptosis with limited or tolerable damage to surrounding normal cells. Several studies have demonstrated that extracts from several herbal medicines or mixtures have an anticancer potential in vitro or in vivo[2][3]. For a long time plants have been provided essentials nutritional values, medicinal properties and notable physiological impression to life and are a rich source of food. The principles key is herbal medicines are comparatively simple, although they are entirely different from the traditional medicines and herbal medicines[4]. India is a great place of medicinal plant and variety of plants extracts are used against diseases in several systems of remedy such as ayurveda, unani and siddha[5]. Some of them have been scientifically analyzed. Medicinal plants are the extremely specific source of life saving medicine for the major part of the world's population. For the treatment of cancer, use of plant products has become a recent interest.

 DOI : 10.18231/2454-9150.2018.0706

II. METHODOLOGY

2.1 Cell Line Screening

HEp-2 cell line is the required cell line and HEp-2 cell line was originated from tumors presented in promulgate cortisonised Cancerous tissue of the larynx of a 56 year old male. These cell lines are found to be indistinguishable to HeLa by STR PCR DNA profiling. Therefore, the HEp-2 cell line should be recognized as derived from HeLa. HeLa contaminant; adherent; Cells contain human papilloma virus. The cells are positive for keratin by immune peroxidase staining. The base medium for this cell line is ATCC-formulated Eagle's Minimum Essential Medium[6].

Fig.1 Rhizome of Ginger

Fig.2 HEp-2 Cell Line

2.2 Preparation of Ginger Extract

Zingeber officinale plant rhizome collected from Biotech Park, Lucknow, dried at room temperature were grind and powdered. 5 gram plant material was loaded in the inert tube of Soxhlet Apparatus and then filtered into round bottom flask containing 200 ml ethanol. The solvent were boiled gently over a water bath using the adjustable rheostat. The extraction was continued for 8 hours and solvent was removed at the reduced pressure with the help of vacuum pump distillation[2].

2.3 Chemicals

EMEM (Eagle`s Minimum Essential Media), DMSO, Trypan blue, Ladder, Trypsin, Isopropanol, 1% Glacial Acetic acid 3,4,5 dimethylthiazolyl2) 2,5diphenyltetrazoliumbromide(MTT), Neural Red Uptake(NRU), Sodium acetate, EtBr, RNase, TE buffer, Proteinase, 40% ethanol, Lysis Buffer.

2.4 Cell Culture

Cell culture media are mixtures of salts, carbohydrates, vitamins, amino acids, metabolic precursor, growth factors, hormones and trace elements. The requirement for these components varies among cell lines, and these differences are partly responsible for the extensive number of medium formulations. Carbohydrates are supplied primarily in the form of glucose. In some instances, glucose is replaced with galactose to decrease lactic acid build-up, as galactose is metabolized at a slower rate. Other carbon sources include amino acids and pyruvate. In addition to nutrients, the medium helps maintain the pH and osmolality in a culture system. The pH is maintained by one or more buffering system; CO2/Sodium bicarbonate, phosphate and HEPES are the most common[7].

Eagle's Minimum Essential Medium (EMEM) was among the first widely used media was formulated by Harry Eagle from his earlier and simpler basal medium (BME). BME was developed for culturing mouse L cells and HeLa cells

Fig.3. Dose of ethanolic extract

2.5 Sub culturing

To maintain cell lines ,first trypsinization process are done or EMEM medium ,new culture are made by transferring some or all cells from a previous culture to fresh growth medium.

2.6 Cell Viability count

Cell viability counts are done by the use of haemocytometer. Blue colour dye which is use in haemocytometer for cell viability counts. Firstly we will mix cell suspension and trypan blue dye then load the mixture into the haemocytometer and viability will be estimate in to the inverted microscope[8].

 DOI : 10.18231/2454-9150.2018.0706

Fig.4. Haemocytometer loaded with trypan blue dye

III. MTT ASSAY

Examination of cell viability and proliferation make the basis for variety of in vitro assays of a cell population's response to external factors. The reduction of tetrazolium salts is a reliable way to examine cell proliferation. The yellow tetrazolium MTT (3 4, 5dimethylthiazolyl2) 2, 5diphenyltetrazolium bromide) is reduced by metabolically active cells, in part by the action of NADH and NADPH dependent dehydrogenase enzymes. The resulting purple formazan within cell can be solubilized and quantified by spectrophotometric means.

The MTT Cell Proliferation Assay measures the rate of cell proliferation and conversely, when cells are metabolically active lead to apoptosis or necrosis, the reduction in cell viability[6][9].

3.1 Schedules for Treatment

Group I: HEp-2 cells without any treatment serve as control.

Group II: HEp-2 cells with final concentration of 20 mg/ml of ginger extract.

Group III: HEp-2 cells with final concentration of 2mg/ml of ginger extract.

Group IV: HEp-2 cells with final concentration of 0.2mg/ml of ginger extract.

Group V: HEp-2 cells with concentration of 0.02mg/ml of ginger extract. Group VI: treat HEp-2 cells with Pacletaxin.

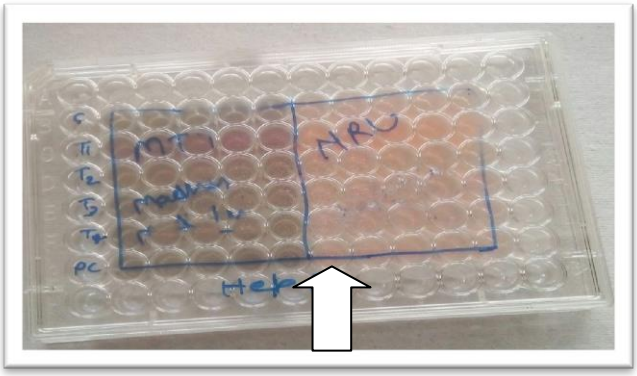

Fig.5. 96 Well plate after the treatment of MTT

3.2 Procedure

Ginger extract dose were prepared mixing with ethanol of different concentration.

Doses were prepared with 100% ethanol extract of clove and weighed 50mg/ml and diluted to 20mg/ml (T_1) concentration and they are further serial diluted in T_2, T_3 and T_4eppendroff tubes. Doses prepared for treatment are of concentration of 1000µg/ml, 100µg/ml, 10µg/ml and 1µg/ml. They are 5µl transferred to each well of 96 well plate as per different column decided for treatment T_1, T_2, $T_3$and T_4. 100µl of 20mg/ml is taken and added with 900µl distilled water and further diluted[10].

IV. NRU ASSAY

The neutral red uptake assay provides a quantitative estimation of the number of viable cells in a culture. It is one of the most used cytotoxicity tests with many biomedical and environmental applications. It is based on the ability of viable cells to incorporate and bind the supravital dye neutral red in the lysosomes[11][12].

4.1 Treatment schedule

Treatment schedule of NRU Assay are same as which are given to the MTT Assay.

4.2 Procedure

HEp-2 cells are seeded into the 96 wells plate then after one day the fresh media is added to the each wells and leave it for 24 h. After, one day NR medium (100µl) is added to the each wells and left for 4 hours. After that NR medium is discarded and 1% Glacial acetic acid in 40% acidified alcohol (100µl) is added to each well. Then we check the absorbance in ELISA reader

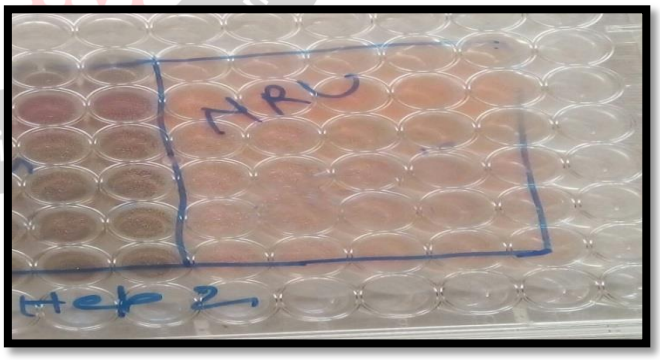

Fig.6. 96 Well plate after treatment of NRU

 DOI : 10.18231/2454-9150.2018.0706

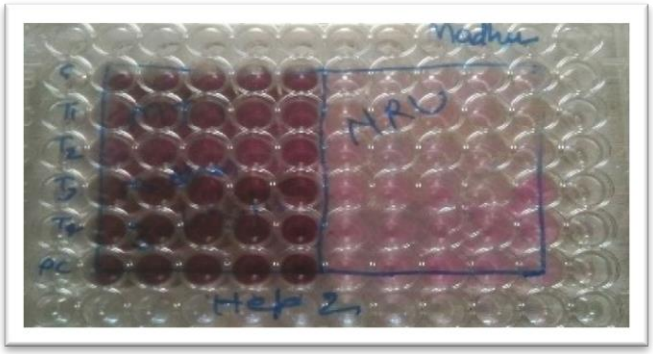

Fig.7. 96 Well plate after Glacial acetic acid treatment

Fig.8. Absorbance test on Micro Titer Plate Reader

V. DNA FRAGMENTATION ASSAY

The detection of apoptosis in cultured cells relies heavily on techniques involving the extraction of nuclear DNA and characterization of such oligonucleosomal ladders by gel electrophoresis. Fragmented DNA remains in the supernatant and can be used directly for agarose gel electrophoresis or quantitation manipulation. DNA fragments in the range of 20–300 kb, an early sign of cellular apoptosis that precedes the generation of low molecular weight oligonucleosomal fragments, are also retained in the supernatant following precipitation. In addition, the percentage of DNA fragmentation in cultured cells detected by this assay exhibits a direct correlation with the percentage of apoptotic nuclei present in these cultures[13][4].

5.1 Treatment Schedule

In this DNA fragmentation assay there are 5 treatment groups, first one is the control with 40% ethanol, second one is the ginger extract at the concentration of 20 mg/ml, third one is 10 time less than the T1 and fourth one is 10 time more less than the T2 and last one is at 0.2mg/ml concentration.

5.2 Procedure for the DNA fragmentation

Harvest cells and centrifuge the appropriate number of cells (2.4×10^5 cells) for 5 min at 300 xg in a 1.5 ml eppendorf tube. Remove the suspention completely and discard, taking care not to disturb the cell pallet.Resuspend the cell pellet in 200µl lysis buffer (with proteinase K) and incubate at 37ºc for 20-45 min. Centrifuge at 12,000x g for 10 min and collect supernatant. Add 50µl, sodium acetate (3M) and 200µl Isopropanol in the collected supernatant and incubate for 2hours to overnight incubation at -20ºc.Centrifuge at 10,000xg for 10 min, Wash the pallet in 70% ethanol. Centrifuge at 10,000xg for 10mins, Discard the supernatant an air dry pallet, dissolve the pellet in 200ul TE buffer. Add 5µl of RNase solution and incubate for 1h at 37ºc. Precipitate the DNA by adding 200µl Isopropanol. Centrifuge at 10,000xg for 10mins. Wash the pellet in 70% ethanol. Centrifuge at 10,000xg for 10mins, Discard the supernatant an air dry pallet. Dissolve the pellet in 200µl TE buffer. Quantify the DNA using nano-drop.

Fig.9. 6 Well plate

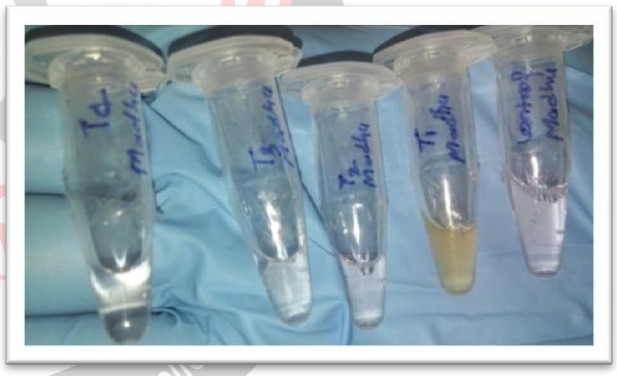

Fig.10. Cells in Eppendorf Tube

	Concentration	Absorbance	260nm	280nm	260/280	260/230
C	43.6	9.174	0.917	10.091	45.871	4.587
T1	49.4	8.097	0.809	8.906	40.485	4.048
T2	39.9	10.025	1.00	11.027	50.125	5.012
T3	205.1	1.950	0.195	2.145	9.751	0.975
T4	28.8	13.888	1.388	15.277	69.444	6.944

Table 1: Nano- Drop readings

	(C) Concentration observed in Nano-drop(ng/µl)	(A) Volume of sample(µl) for 400 ng [(1/C)* 400]	(B) Volume of dye (µl) [A/10]	Total volume [A+B]

 DOI : 10.18231/2454-9150.2018.0706

C	43.6	10	1	11
T1	49.4	9	1	9
T2	39.9	11	2	12
T3	205.1	2	1	3
T4	28.8	14	2	16

Table 2: Calculation for loaded sample in wells of gel

VI. RESULT

Cell Culture

There were 2.0×10^5 cell/ml in a stock solution and the viability was 100%.

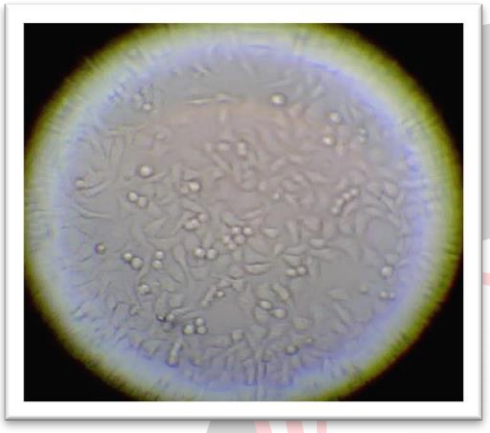

Fig.11. Cell Growth

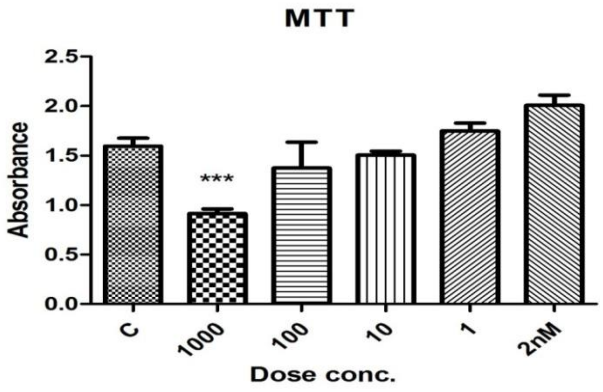

Fig.12. MTT result shows 99.9% cytotoxicity

HEp-2 cells were treated with different concentrations of ginger extract and the viability was measured by the MTT reduction assay, among the four concentrations tested, the 1000µg showed the maximum growth inhibition. The effect was further confirmed by results obtained from the contents of DNA.

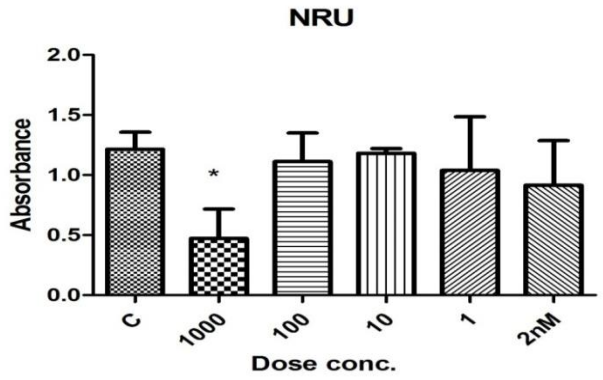

Fig.13. NRU result shows 95% cytotoxicity

Cytotoxicity in HEp – 2 cells induced by exposure to different concentration of ginger is determined by NRU Assay. The columns represent the percentage of viability in HEp-2 cells. Cell viability is shown as from at least four separate experiments done. The values are represented as mean ± S.D. * P < 0.0001 versus control.

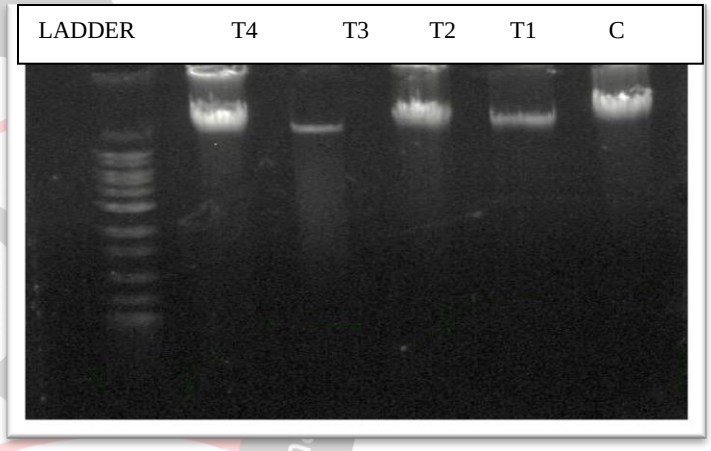

Fig.14. DNA Band

DNA fragmentation Assay perfectly determines the cytotoxicity of the ginger on HEp-2 cell line. Decreasing the concentration of ginger extract in every well of the cell plate has some cytotoxic effect on HEp -2 cell line. DNA got fragmented and separated in electrophoresis gel. Long lines in the gel represent the high concentration of fragmented DNA. Control has very less DNA fragment and other have high DNA fragment. T1 have more DNA fragments than T2, T3 and T4. In the following way there is the increase in the order of the DNA fragments: C < T4 < T3 < T2 < T1. Ethanolic extract had been used in various anti-cancerous experiments.

VII. CONCLUSION

In conclusion, results from the present study suggest that the crude ethanolic extract of ginger exhibited in vitro cytotoxic , anti-oxidant, and apoptotic activities in HEp-2 cell line. This could be attributed to phytochemicals present

 DOI : 10.18231/2454-9150.2018.0706

in the ginger extract like 6-gingerol and 6-paradol.Even through further studies are needed to confirm these properties of ginger in vivo also.

REFERENCE

[1] S. Prasad and A. K. Tyagi, "Ginger and Its Constituents: Role in Prevention and Treatment of Gastrointestinal Cancer," Gastroenterol. Res. Pract., vol. 2015, pp. 1–11, 2015.

[2] V. Vijaya Padma, S. Arul Diana Christie, and K. M. Ramkuma, "Induction of apoptosis by ginger in HEp-2 cell line is mediated by reactive oxygen species," Basic Clin. Pharmacol. Toxicol., vol. 100, no. 5, pp. 302–307, 2007.

[3] R. Hu et al., "6-Shogaol Induces Apoptosis in Human Hepatocellular Carcinoma Cells and Exhibits Anti-Tumor Activity In Vivo through Endoplasmic Reticulum Stress," vol. 7, no. 6, pp. 1–11, 2012.

[4] T. Plengsuriyakarn et al., "A study on cytotoxicity, toxicity and anticancer activity of zingiber officinale roscoe against cholangiocarcinoma," Res. J. Pharmacol., vol. 6, no. 3, pp. 35–47, 2012.

[5] W. Koch et al., "Application of chromatographic and spectroscopic methods towards the quality assessment of ginger (Zingiber officinale) rhizomes from ecological plantations," Int. J. Mol. Sci., vol. 18, no. 2, pp. 1–15, 2017.

[6] American Type Culture Collection, "MTT Cell Proliferation Assay Instruction Guide," Components, vol. 6597, pp. 1–6, 2011.

[7] M. Borowiak et al., "Photoswitchable Inhibitors of Microtubule Dynamics Optically Control Mitosis and Cell Death," Cell, 2015.

[8] K. S. Louis and A. C. Siegel, "Mammalian Cell Viability," vol. 740, no. 1, pp. 7–12, 2011.

[9] S. Rahman, F. Salehin, and A. Iqbal, "In vitro antioxidant and anticancer activity of young Zingiber officinale against human breast carcinoma cell lines," BMC Complement. Altern. Med., 2011.

[10] O. Germaniuk, "MTT Assay Protocol," p. II.E.4, 2010.

[11] U. S. P. H. Service, "for the NHK Neutral Red Uptake Cytotoxicity Test A Test for Basal Cytotoxicity for an In Vitro Validation Study Phase III Prepared by The National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICE," Environ. Prot., pp. 1–20, 2003.

[12] C. K- and C. Store, "Neutral Red Cell Cytotoxicity Assay Kit," no. 408, pp. 1800–1801, 1801.

[13] Y. a. Ioannou and F. W. Chen, "Quantitation of DNA fragmentation in apoptosis," Nucleic Acids Res., vol. 24, no. 5, pp. 992–9sss93, 1996.

 DOI : 10.18231/2454-9150.2018.0706