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In vitro anti-cancer activity of ethanolic extract of curcumin longa (turmeric) in HEp-2 cell lines

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Abstract— Curcumin, an active constituent of Curcuma longa, is responsible for the anticarcinogenic activities which are mediated through multiple mechanisms. The cytotoxicity tests were performed in this experiment which defines the upper limit of the extract concentration, which can be used in subsequent anticancer studies. In vitro cytotoxic activity of turmeric extracts against Hep2 cancer cell lines were evaluated in this experiment. Hep2 cells were treated with different concentration of plant extracts and morphological changes were observed under inverted microscope. First, the cell viability check was done with the trypan blue and then different concentrations of turmeric extracts were treated with MTT (3 4, 5dimethylthiazolyl2)2,5diphenyltetrazoliumbromide) and NRU (Neutral Red Uptake). The test sample showing cytotoxicity of more than 97% at 1000µg/ml, were considered to be less active at minimum concentration. At last DNA fragmentation method was used to determine the induction of apoptosis inside the cell.

Keywords— MTT (3 4, 5dimethylthiazolyl2)2,5diphenyltetrazoliumbromide), NRU (Neutral Red Uptake), Anti-cancer activity, Curcumin longa, apoptosis, ethanolic extracts, cytotoxicity

INTRODUCTION

Cancer is a leading cause of death worldwide and had accounted for 7.9 million deaths (approximately 13% of all deaths) in 2007. Most drugs currently available for the treatment of cancer have limited potential, because they are highly toxic, inefficient in treating cancer, or highly expensive. Treatments without these disadvantages are needed. Hence, the identification and synthesis of novel, efficient and less toxic anticancer agents remains an important and challenging task for the cancer treatment. Use of plant extracts as medicine for cancer treatment is certainly the effective method and dozens of plant based products have been reported for cancer treatment progress. Plant based products such as curcumin occupied significant role against cancer, microbial infections and other inflammatory diseases. Due to its wide range of biological and pharmacological effects and lack of toxicity, curcumin was selected for this study. Curcumin is a naturally occurring yellow pigment isolated from the rhizome of the perennial herb Curcuma longa which has been cultivated for centuries in several Asian countries.

Curcumin is known for its antioxidant, anti-inflammatory, anti-fatigue, antiparasitic, antiallergic, anti-microbial, anti-mutagenic and anticancer properties. It exhibits wide therapeutic potential due to the multi-targeting nature against variety of different cancers including leukemia, gastrointestinal cancers, genitourinary cancers, breast cancer, etc. Curcumin has been shown to suppress transformation, proliferation, and metastasis of tumors. It also inhibits proliferation of cancer cells by arresting them in various phases of the cell cycle and by inducing apoptosis. It is obvious that curcumin's multitargeting ability may be the key to its therapeutic potential against cancer. An ethanolic extract of turmeric, as well as an ointment containing curcumin, is reported to produce remarkable symptomatic relief in patients with external cancerous lesions. It is now proved that the antioxidants present in turmeric neutralize carcinogenic free radicals.

Curcuminoids possess anti-carcinogenic property due to their oxygen radical-scavenging property. In a comparative study of curcuminoids for their free radical-scavenging activity, turmeric is found to be the most potent free radical scavenger, followed by dimethoxycurcumin and bis-demethoxy curcumin. Acetyl curcumin was found inactive. Reports showed the use of turmeric preparations in the treatment of cancer. In the course of a search for antitumor agents, the extract of turmeric was found to be effective in inducing apoptosis or programmed cell death (PCD) in human myeloid leukemia cells (HL - 60). Curcuminoids protect the normal human keratinocytes from hypoxanthine/xanthine oxidase injury. Further, they proposed that since curcuminoids synergistically

inhibited nitrobluetetrazolium reduction, a decrease in superoxide radical formation, leading to lower levels of cytotoxic hydrogen peroxide, might explain the protective effect.

REVIEW OF LITERATURE

Cancer is the uncontrolled cell proliferation and production of a growing mass cell. Cancer cells can break away and spread to other body parts, particularly in the blood stream and lymphatic system, causing metastatis. Over the years, different approaches have been employed and are still in use, individually or in combination, in the treatment of cancer. These include chemotherapy, radiotherapy, surgery and immunotherapy. Plants have a long history of use in the treatment of cancer and the interest in nature as a source of potential chemotherapeutic agent continues. The present day research and development tailored towards the discovery of new anti proliferative agents from natural products has been buoyed by improvement in science and technology of anticancer drug discovery. Cancer initiation has been produced by oxidative stress and chronic inflammation. Inflammation acts a key regulator in promotion of these initiated cells, possibly by providing them with proliferating signals and by preventing apoptosis. The role of inflammation in tumor induction and subsequent malignant progression has been investigated. Inflammatory response produces cytokines which act as growth and/or angiogenic factors leading transformed cells to proliferate and undergo promotion.

Turmeric (Curcuma longa) is extensively used as a spice, food preservative and coloring material in India, China and South East Asia. It has been used in traditional medicine as a household remedy for various diseases, including cough, diabetic wounds hepatic disorders. Curcumin (diferuloylmethane), the main yellow bioactive component of turmeric has been shown to have a wide spectrum of biological actions and isolated from the rhizome of the plant Curcuma longa. These include its anti-inflammatory, antioxidant, anticarcinogenic, anticoagulant, antifertility, antidiabetic, antibacterial, antifungal, antiprotozoal, antiviral, antifibrotic, antivenom, antiulcer, hypotensive and hypocholesteremic activities. Its anticancer effect is mainly mediated through induction of apoptosis. Safety evaluation studies indicate that curcumin is well tolerated at a very high dose without any toxic effects. Thus, curcumin has the potential for the development of modern medicine for the treatment of various diseases. Curcumin longa, botanically related to ginger (Zingiberaceae family), is a perennial plant having a short stem with large oblong leaves and bears ovate, pyriform or oblong rhizomes, which are often branched and brownish-yellow in color. Curcumin (diferuloylmethane) (3–4%) is responsible for the yellow color.

It exhibits wide therapeutic potential due to the multi-targeting nature against variety of different cancers including leukemia, gastrointestinal cancers, genitourinary cancers, breast cancer, etc. Curcumin has been shown to suppress transformation, proliferation, and metastasis of tumors. It also inhibits proliferation of cancer cells by arresting them in various phases of the cell cycle and by inducing apoptosis. It is obvious that curcumin's multitargeting ability may be the key to its therapeutic potential against cancer. Curcumin induced apoptosis mainly involves the mitochondria-mediated pathway in various cancer cells. Curcumin causes Hep2 cells to develop characteristic features of cell shrinking, rounding and partial detachment, thus demonstrating the lobulated appearance of apoptotic cells. Curcumin was found to inhibit the generation of ROS including superoxide dismutase and hydrogen peroxide in peritoneal macrophages. It inhibits lipo-polysaccharide and interferon-γ-induced production of nitric oxide in macrophages and inhibition of inducible nitric oxide synthase gene expression in isolated BALB/c mouse peritoneal macrophages. Curcumin prevents phosphorylation and degradation of inhibitor κ B α, thereby blocking NF-κB activation which down regulates iNOS gene transcription. Deregulatory imbalances between adaptive and innate immunity results in chronic inflammation, is associated with epithelial tumorigenesis, through the NF-κB activation. Curcumin was found to inhibit cell proliferation and cytokine production by inhibiting NF- κB target genes involved in this mitogen induction of Tcell proliferation, interleukin and nitric oxide generation. Reduction induced over expression of cytokines, such as IL-10, IL-6, and IL-18, is accompanied by NF-κB induction which is controlled by and inhibited by curcumin. Curcumin has been demonstrated to increase expression of conjugation enzymes (phase II). These have been shown to suppress ROS-mediated NF-κB, AP-1 and mitogen activated protein kinases (MAPK) activation.

For the last few decades, extensive work has been done to establish the biological activities and pharmacological actions of turmeric and its extracts. Curcumin was first isolated in 1815 and its chemical structure was determined by Roughley and Whiting in 1973. It has a melting point at 176–177° C; forms a reddish-brown salt with alkali and is soluble in ethanol, alkali, ketone, acetic acid and chloroform. Turmeric was already proved beneficial in various types of cancers like duodenal tumors, tongue carcinoma, colon cancer, human breast cancer cells in-vitro, mammary tumor in vivo.

Fig. 1. Curcumin longa

Curcumin has been shown to promote apoptosis in certain cancer cell lines and to inhibit telomerase activity, an important factor in tumorigenesis. One possible mechanism for the induction of tumor cell death is through the generation of reactive oxygen intermediates. Although curcumin is the acknowledged active principal in turmeric, the oleoresin of turmeric (after extraction of curcumin) also was found to have anti mutagenic properties, thought to be mediated through its antioxidant action.

The anti-inflammatory properties of curcumin are thought to be due in part to suppression of prostaglandin synthesis. Prostaglandin synthesis from arachidonic acid is catalyzed by two isoenzymes: COX-1 and COX-2, both found in colon tumors of rodents and humans. Goel et al found that curcumin significantly inhibited expression of COX-2 in human colon cancer cell and in COX-2 non-expressing cell lines, without altering the expression of COX-1. This is an important benefit of curcumins since chronic use of nonsteroidal anti-inflammatory drugs (NSAIDs) and non-specific inhibition of COX-1 lead to undesirable gastrointestinal and renal side effects. Curcumin also was shown by Mahady et al to inhibit the growth of Helicobacterpylori, a group carcinogen, as a possible explaining mechanism for its role in prevention of gastric and colon cancers in rodents. Animal studies involving rats and mice, as well as in vitro studies utilizing human cell lines, have demonstrated curcumin's ability to inhibit carcinogenesis at three stages: tumor promotion, angiogenesis, and tumor growth. In two studies of colon and prostate cancer, curcumin inhibited cell proliferation and tumor growth. Turmeric and curcumin are also capable of suppressing the activity of several common mutagens and carcinogens in a variety of cell types in both in vitro and in vivo studies. The anticarcinogenic effects of turmeric and curcumin are due to direct antioxidant and free-radical scavenging effects, as well as their ability to indirectly increase glutathione levels, thereby aiding in hepatic detoxification of mutagens and carcinogens, and inhibiting nitrosamine formation

CELL LINE AND ITS ROLE IN SCREENING

Hep2 cell line is the required cell line and it was: Originated from tumors produced in irradiated-cortisonised weanling rats after injecting with epidermoid carcinoma tissue from the larynx of a 56 year old male. This cell line was found to be indistinguishable from HeLa by STR PCR DNA profiling. Therefore, the cell line should be considered as derived from HeLa. HeLa contaminant; adherent; Cells contain human papilloma virus. The cells are positive for keratin by immunoperoxidase staining. The base medium for this cell line is ATCC-formulated Eagle's Minimum Essential Medium (EMEM).

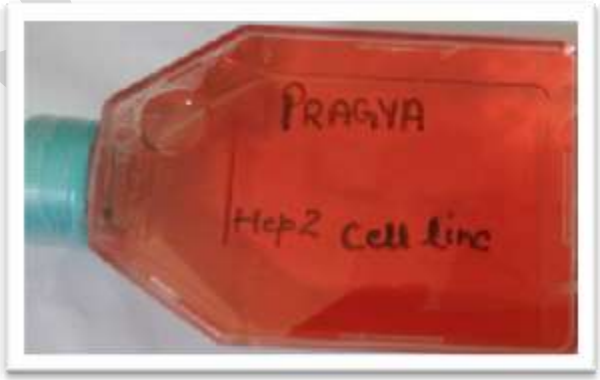

Fig. 2. Cultured hep2 cell line

The purpose of this study was to evaluate, in vitro the efficacy of turmeric extract as anti cancer agent in hep2 cell line. Most drugs currently available for the treatment of cancer have limited potential, because they are highly toxic1, inefficient in treating cancer, or highly expensive. Treatments without these disadvantages are needed.

Hence, the identification and synthesis of novel, efficient and less toxic anticancer agents remains an important and challenging task for the cancer treatment. Use of plant extracts as medicine for cancer treatment is certainly the effective method and dozens of plant based products have been reported for cancer treatment progress.

Since the plant based products have the natural multi-targeting ability as well as inexpensive and is safe as compared to synthetic agents. Among them, plant based products such as curcumin occupied significant role against cancer, microbial infections and other inflammatory diseases.

MATERIALS AND METHODS

F. Plant Extraction

Curcuma longa plant rhizome collected from Biotech Park Lucknow, dried at room temperature were grind and powdered. The plant material 6g was loaded in the inert tube of soxhlet apparatus and then filtered into round bottom flask containing 200ml acetone. The solvent were boiled gently over a water bath using the adjustable rheostat. The extraction was continues for 8 hours and solvent was removed at the reduced pressure with the help of vacuum pump distillation.

G. Chemicals

Trypan blue, Ladder

Trypsin

Eagle's Minimum Essential Media

DMSO

1% Glacial Acetic Acid

MTT (3 4, 5 dimethylthiazolyl2)2,5diphenyltetrazoliumbromide)

NRU (Neutral Red Uptake)

Isopropanol

Lysis Buffer

40% ethanol

RNAase

Proteinase

Sodium acetate

TE buffer

EtBr

H. Cell Culture

Cell culture media are complex 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.

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 there have numerous variations on EMEM formula for different applications. EMEM contains balanced salt solution, non essential amino acids, and sodium pyruvate.

I. Passaging

To culture our cell lines the trypsinization process will be done first and or EMEM media will be added. After the centrifugation the supernatant will be discarded and in the pellet again the complete media will be added and two other flask will be prepared process is called subculturing.

J. Cell viability count

It is a blue colour dye which is used in the haemocytometer to estimate the cell viability. To estimate the cell viability first we mix the cell suspension and the trypan blue dye then load the mixture into the haemocytometer and estimate the viability the cell into the inverted microscope.Case-Study.

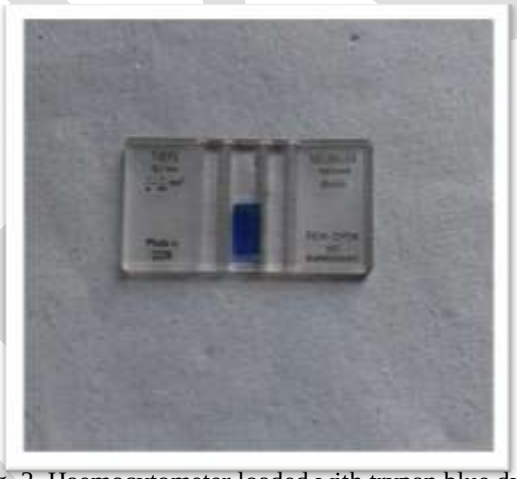

Fig. 3. Haemocytometer loaded with trypan blue dye

K. MTT Assay

Measurement of cell viability and proliferation forms the basis for numerous in vitro assays of a cell population's response to external factors. The reduction of tetrazolium salts is now widely accepted as a reliable way to examine cell proliferation. The yellow tetrazolium MTT (3 4, 5 dimethylthiazolyl2)2,5diphenyltetrazoliumbromide) is reduced by metabolically active cells, in part by the action of dehydrogenase enzymes, to generate reducing equivalents such as NADH and NADPH. The resulting Intracellular purple formazan can be solubilized and quantified by spectrophotometric means. The MTT Cell Proliferation Assay measures the cell proliferation rate and conversely, when metabolic events lead to apoptosis or necrosis, the reduction in cell viability.

L. Treatment schedule

In my experiment there are six treatment groups first one is control group in which 40% ethanol is added, second one is T1 group in which 20mg/ml concentration of turmeric extract will be added, third one is T2 group in which 2mg/ml concentration of turmeric

extract will be added, fourth group is T3 group in which 0.2 mg/ml concentration of turmeric extract will be added, fifth group is T4 group in which 0.02mg/ml concentration of turmeric extract will be added and last group is positive control group in 2µl paclitaxin will be added which is a anticancer drug.

Fig. 4. Stock solution for the treatment

M. Procedure of MTT Assay:

100µl cell suspension will be added into 96 well plates and leave it for one day. When the cell will grow properly the new media will be added and the treatment will be given to the cell lines. After 20 hours the MTT will be added and leave it for 2 h. After 2 h the yellow color MTT becomes blue in color then the whole media will be taken out properly without damaging the present cells into the well plate and then 100 µl DMSO will be added. Mix the DMSO and cells properly and take the reading into the ELISA reader.

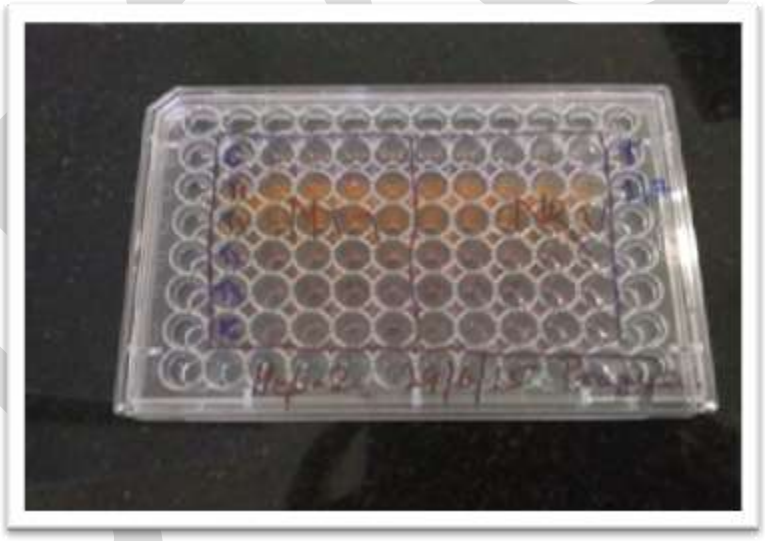

Fig. 5. 96 Well plate after the treatment of MTT

N. 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 supra vital dye neutral red in the lysosomes. Most primary cells and cell lines from diverse origin may be successfully used. Cytotoxicity is expressed as a concentration dependent reduction of the uptake of NR after chemical exposure, thus providing a sensitive, integrated signal of both cell integrity and growth inhibition.

Cells are seeded in 96-well tissue culture plates and are treated for the appropriate period. The plates are then incubated for 2 h with a medium containing neutral red. The cells are subsequently washed, the using a spectrophotometer.

O. Treatment schedule

The same treatment is given to the NRU also which will be described above in MTT ASSAY.

P. Procedure used for NRU ASSAY

100µL cell suspension will be added into the 96 well plate and leave it for overnight. Next day fresh media will be added and the treatment will be given as described above and leave it for 20 hours. Then the NR will be added and leave it for 4 hours and then the whole medium will be taken out properly without disturbing the cell lines and then 100µl, 1% Glacial Acetic Acid in 40% acidified alcoh ol and reading will be taken into the ELISA reader.

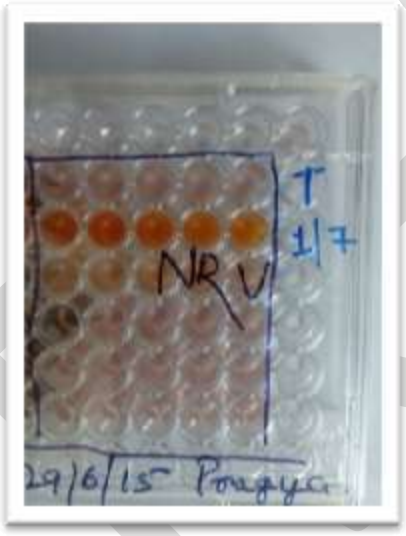

Fig. 6. 96 Well plate after the treatment of NRU

Fig. 7. While recording the absorbance into the ELISA reader

Q. DNA Fragmentation Assay

It is the separation or breaking of DNA into pieces. It can be done intentionally by laboratory personell or by cells. DNA fragmentation was first documentated by Williamson in 1970 when he observed discrete oligomeric fragments occurring during cell death in primary neonatal liver cultures. He described the cytoplasmic DNA isolated from mouse liver cells after culture as

characterized by DNA fragments with a molecular weight consisting of multiples of 135 Kda. This finding was consistent with the hypothesis that these DNA fragments were a specific degradation product of nuclear DNA.

R. Treatment schedule

In this experiment there were 5 treatment groups, first one is control in which 40% ethanol will be added, second one is turmeric extract at the concentration of 20 mg/ml, third one is 10 times less than the T1 i.e. 20 mg/ml and the fourth one is ten times more less than the T2 i.e. 2 mg/ml and the last one is at the concentration of 0.2 mg/ml.

S. Procedure used for the DNA fragmentation

The precultured cells were first trypsinized then plated into the 6 well plate (1.5 ml/well) and leave it for overnight. Next day media will be changed and the treatment will be provided as discussed above and leave it for 24 h. Then the media will be discarded and 200 µl lysis buffer will be added for the cell scrapping and washing and collect it into the 5 different eppendrofs, then RNAase will be added (2µl) and incubate it for 1h after the time duration Proteinase (2µl) will be added and again incubate it for 1h. After all this treatment centrifuge the sample at 10,000 rpm for 10 min. Then take the supernatant and add sodium acetate (50µl) and Isopropanol (200µl). Now the strands of DNA will be isolated again centrifuge it, now discard the supernatant and add 70% ethanol Again centrifuge it discard the supernatant and dry pellet, then add t he TE buffer and take the re ading into the nano drop.

Fig. 8. 6 Well plate after the treatment

Fig. 9. Eppendrofs containing lysis buffer

T. Agarose gel electrophoresis

First 30mg agarose was dissolved in 30ml distilled water and 75µl TE buffer. Boil the solution until it became transparent then add0.5µl and pour 15ml agarose into the casting tray. After 15-20 min place you're casting tray into the electrophoresis unit and load your sample into the wells. When then gel will run take the reading into gel documentation system.

	Conc.(ng/µl)	sample volume for400ng DNA	Amount of loading Dye	Amount to loaded into gel
C	87.2	4.6	0.458716	5.0
T1	40.9	9.8	0.977995	10.8
T2	33.7	11.9	1.186944	13.1
T3	23.8	16.8	1.680672	18.5
T4	90.9	4.4	0.440044	4.8

Fig. 10. Reading of sample loaded in gel electrophoresis

Fig. 11. Gel casting

Fig. 12. Gel electrophoresis unit

RESULTS

A. For cell viability count

100% viability was found

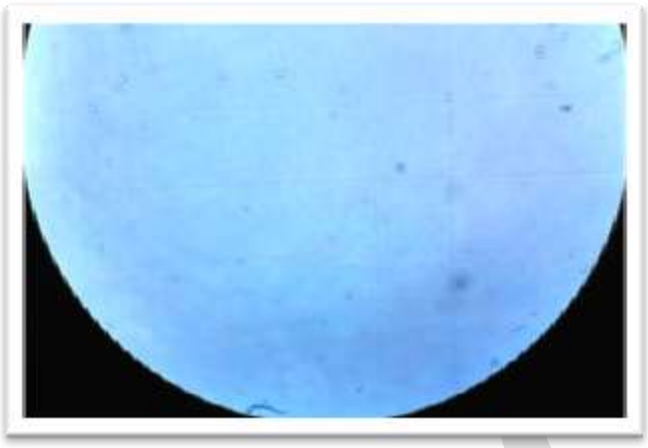

Fig. 13. Cells under the inverted microscope

B. MTT Assay

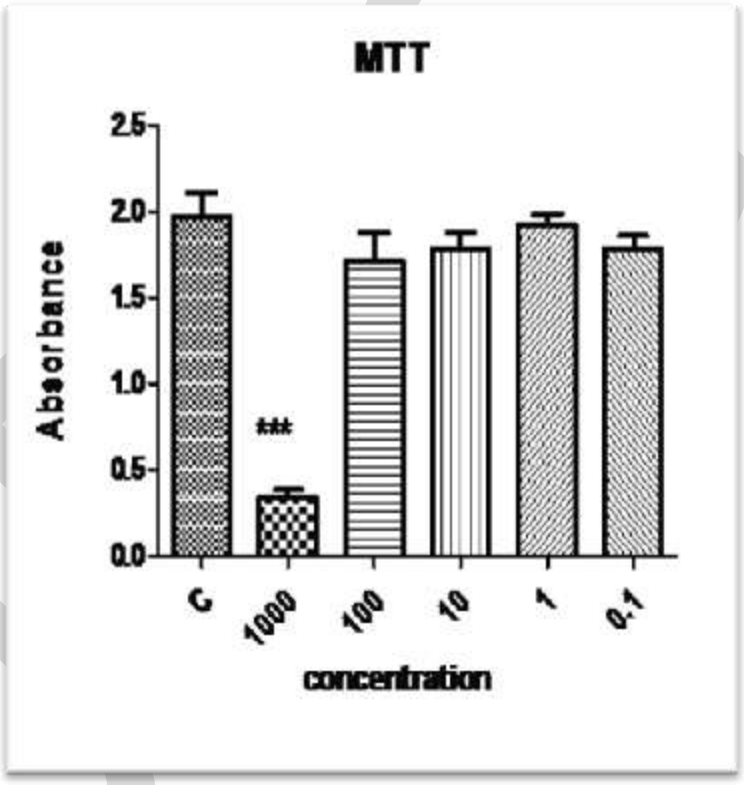

Fig. 14. Graph Showing MTT result

Extract of turmeric was treated with MTT at different concentration and maximum significant death was found at 1000µg/ml as compared to low concentration. Very less amount of viable cells were detected at this concentration which shows the maximum inhibition concentration. While decreasing the concentration of curcumin to and the viability of the cell showing negligible amount of cell death and minimum lethal dose.

C. NRU Assay

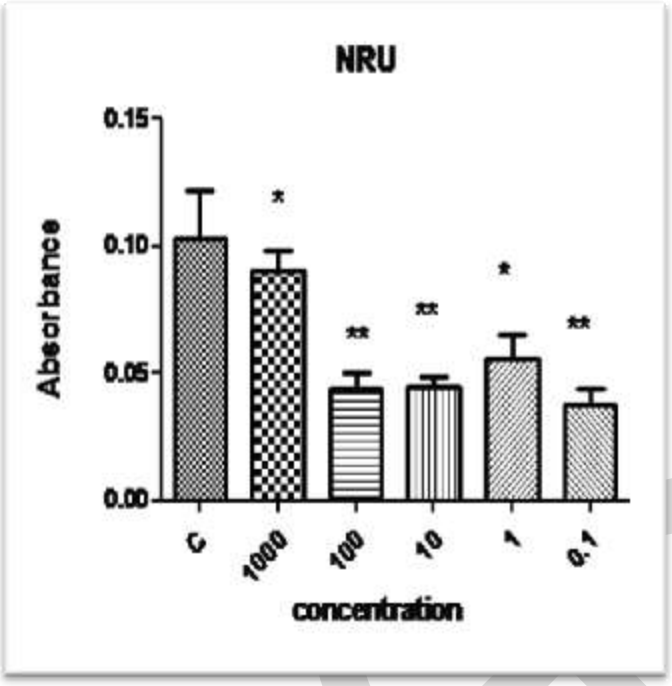

Fig. 15. Graph showing NRU result

Extract of turmeric when treated with NRU then significant death of cells was observed at low concentrations also.

D. DNA Fragmentation assay

Fig. 16. While taking the reading into the nanodrop spectrophotometer

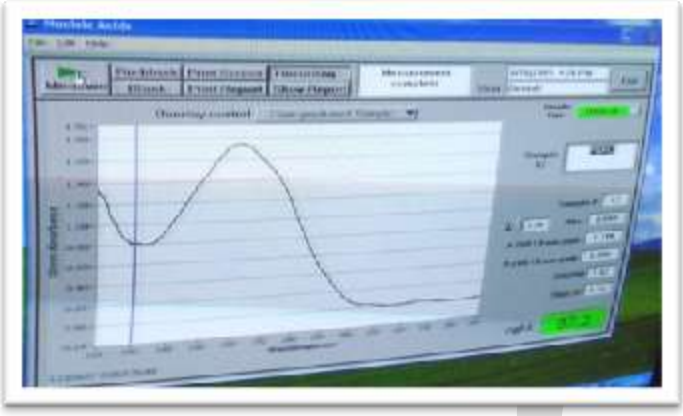

Fig. 17. Nano drop reading for control

Control contains the pure DNA there is no contamination of the RNAase and Proteinase because the 260/280 ratio is 1.82

E. Nano drop reading

Table 1. Data of DNA samples in Nano drop spectrophotometer

concentration	ABSORBANCE	260nm	280nm	260/280nm	260/230nm
C	0.808	1.744	0.956	1.82	2.16
T1	0.394	0.818	0.448	1.82	2.08
T2	0.332	0.674	0.366	1.84	2.03
T3	0.251	0.476	0.285	1.67	1.90
T4	0.201	0.344	0.199	1.62	1.80

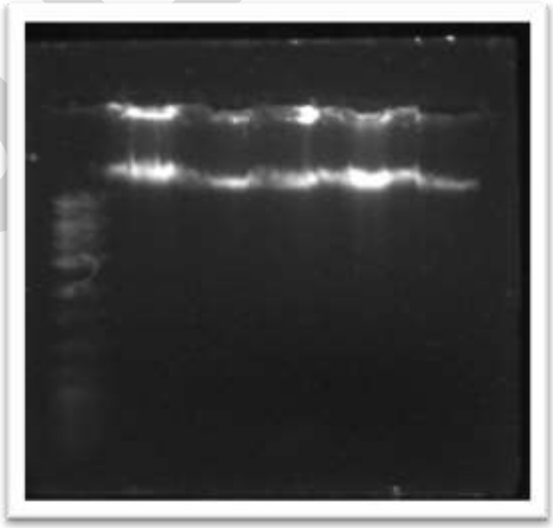

Fig. 18. DNA bands of gel documentation

DISCUSSION

Previous studies has indicated that the turmeric has the ability to induce the apoptosis in very cancerous cell lines like HL-60, HELA etc. Curcumin is the principal component present in turmeric which posses the anticarcinogenic property. The cytotoxicity study was carried out with the Hep2 cell lines at different concentrations which indicated that it inhibits the cell growth at 1 mg/ml. Furthermore, DNA fragmentation assay also indicated the fragmentation of DNA at the same dose.

CONCLUSION

Based on the reported results, it may be concluded that pure curcumin and the crude ethanolic extract have great potential in the prevention and cure of cancer. At higher dose of curcumin on cancerous cell line apoptosis has been started inside the cells and the maximum reduction of growth shows. We therefore conclude that plant extracts showed selective in-vitro cytotoxicity against Hep2 cancer cell lines and plants were found to be highly effective against various cancerous cells. However, there is further in-vivo studies are also needed to ensure the anti-tumorogenic property of turmeric in cancerous cell line.

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