Original Research Article

Antitumour Activity of *Xanthium strumarium* L. on Human Cervical Cancer HeLa Cells.

ABSTRACT

Aim: The present investigation was aimed at assessing the antiproliferative and apoptotic potential of the hydroalcoholic extract of *X. strumarium* on HeLa cell line.

Study design: Antitumour activity of *Xanthium strumarium* was checked by testing against cultured HeLa cells.

Place and duration of the study: Department of Zoology BMTC and Human Genetics, University School of Sciences, Gujarat University,Ahmedabad, Gujarat, India between January 2012 and October 2012.

Materials and Methods: Antiproliferative activity was evaluated by MTT assay in a dose and time dependant manner after treatment with the *Xanthium* extract. Trypan blue viability assay was also performed. Apoptosis induction in the cells post treatment was determined by DNA fragmentation assay by diphenylamine method, Agarose gel electrophoresis and Acridine orange / Ethidium bromide dual staining. The results were compared to that of untreated cells. Effect of treatment on HeLa cellular protein level was determined by colourimetric assay. Also, the proteins were isolated and analyzed on polyacrylamide gel.

Results: The extracts inhibited growth and proliferation of HeLa cells through induced cell death, which was dose and time dependant. The IC_{50} value was found to be 34.64 μg/ml after 48 hours of treatment. The induction of DNA fragmentation and the increase in number of apoptotic cells post treatment suggest the possibility of apoptosis induction. A significant decrease in protein level was also observed.

Conclusion*:* Although further study of the chemotherapeutic effect of this plant is required, the results raise the possibility that the extract of *Xanthium strumarium* is a potent chemotherapeutic agent for treatment of various cancers.

Key words: *Xanthium strumarium*, HeLa cell line, Antiproliferative, Apoptotic.

1. INTRODUCTION:

Cancer is a genetic disease characterized by uncontrolled cell growth in the absence of cell cycle regulation. Aberrant cell cycle regulation can arise as a consequence of DNA damage. Under normal physiological conditions the uncontrolled growth of damaged cells is restricted by apoptosis. However these cells can escape the regulatory mechanisms of apoptosis as a result of secondary mutations to genes that regulate apoptosis. This DNA damage can be a result of several environmental factors such as stress, smoking, pollution, diet, toxins and endogenous processes such as errors in replication of DNA and chemical instability of certain DNA bases [1]. The incidence of cancer is increasing worldwide and it is the single most common cause of death in both developed and developing countries. In 2008 approximately 12.7 million cancers were diagnosed and 7.6 million people died of cancer worldwide [2]. Cancers as a group account for approximately 13% of all deaths each year [3]. In India, around 5,55,000 patients died of cancer in 2010, according to estimates published in *The Lancet* today [4]. Treatment options for cancer majorly include surgery, chemotherapy and radiation therapy which have their own limitations and side effects. Approximately five decades of systemic drug discovery and development have established a respectable armamentarium of useful chemotherapeutic agents [5, 6] as well as a number of important successes in the treatment and management of human cancer [7]. Nevertheless, the need for more effective antineoplastic agents remains. The most common tumors of the adult are

resistant to available antineoplastic drugs [5, 8] and the majority of these agents have only limited anti-solid tumor activity [5, 6]. Furthermore, these agents have little impact on survival rates [8].

As the conventional cancer therapies fail to completely fulfill the criteria for a successful cancer therapy due to certain limitations and side effects, the use of naturally developed anticancer agents has evolved as an alternative safe, low-cost and convenient one. Since extracts prepared from the whole plant or its part(s) are non-toxic to humans, scientists have devised a novel strategy to utilize it by fortifying with an optimized amount of the purified bioactive agent(s) for a selective chemosensitization of the tumor cells. Hence, such non-toxic chemoprevention agents from natural resources are being studied by researchers for this purpose. Natural compounds have the capacity to prevent, inhibit or reverse carcinogenesis or prevent the development of invasive cancer with limited side effects. Hence, thousands of herbal and traditional compounds are being screened worldwide to validate their use as anti-cancer drugs.

Our aim was to develop new and cost effective drugs against cancer. Taking this into consideration, we have selected plant *Xanthium strumarium* in our study. It has been used priorly as a cure for leucoderma, biliousness, epilepsy, salivation, fever and poisonous bites of insects. The plant of *Xanthium* yields xanthinin which acts as a plant growth regulator [9]. Two sesquiterpene lactone glycosides and three kaurene glycosides isolated from Xanthium species closely related to carboxyatractyloside and atractyloside, are 3′, 4′ didesulphatedcarboxyatractyloside and 3′, 4′-didesulphatedatractyloside [10], xanthanol, isoxanthanol and their C-4 epimers [11]. Polyphenol 1,3,5-tri-*O*-caffeoylquinic acid, accompanied by 3,5- di-*O*-caffeoylquinic acid, has been isolated from the fruit of *Xanthium strumarium* [12]. Xanthiazone and Caffeic acid have also been found from *Xanthium strumarium* [13]. There have been reports of *in vivo* antitumour activity of the plant in transplantable tumors in mice [14].

2. MATERIALS AND METHODS

2.1. Plant extract

Fruits of *Xanthium* were collected and authenticated by Botany Department, Gujarat University (Certificate No. GU/Bot/2013 Dated. 25/10/2013). Fruits were washed and shade dried under ambient temperature. After dried, the plants were sliced and ground using a steel blender. 50 g defatted plant material was capsulated in filter paper and kept in the thimble, 500 ml solvent (water : methanol) (70 : 30) was added into the flask and continuous extraction was carried out in the soxhlet apparatus for 72 hours at 60-70° C till the colour in the siphon became colourless. The extract was dried at 60° C under fumehood. 2-5% yield was obtained. The yield collected after drying was weighed and stored at 4°C until further use in sterile containers, as the hydroalcoholic extract.

The plant extracts for dosing were prepared with the concentrations 1 mg/ml in the Culture media with 5% DMSO and used further with various dilutions. Initial tests were carried out to check the toxicity of DMSO and hence the cells were incubated with media containing 5% DMSO. As compared with cells incubated with only media, there was little or no difference observed in loss of cell number or no other toxic effect was observed. Hence, DMSO treatment was not considered further for various assays.

2.2. Cancer cell culture

For cancer cell culture, HeLa cervix cell line ATCC®CCL-2™ was obtained from National Centre for Cell Science (NCCS), Pune. Cells were cultured in Minimum essential medium (MEM) with 10% fetal bovine serum and antibiotic antimycotic solution. All these supplements were procured from Hi-Media Laboratories. Cell cultures were maintained in a CO_2 incubator at 37°C. All the assays were carried out in the growth phase of the cells. For all the assays a definite number of cells i.e. 2 x 10^6/ ml were used.

2.3. MTT Assay

The effect of the extracts on cell growth (metabolically active cells) was determined by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide] assay as described by modified method of Mosmann (1983)[15]. The cells were seeded in the 96 well plate (the final number before treatment was 2 x 10^6/ ml) and treated with plant extract with the concentrations 50 µg/ml, 25 µg/ml, 12.5 µg/ml respectively for 24, 48 and 72 hours for initial screening. 0.5% (v/v) MTT was added to the culture

medium at the end of incubation period (at each interval). The cells were incubated for a further 2 h at 37℃. The yellow MTT dye is reduced by succinic deh ydrogenase in the mitochondria of viable cells to purple formazan crystals, which are then solubilised in DMSO. The absorbance was read at 540 nm against a DMSO blank. Cell numbers per well were extrapolated from calibration curves for individual cell lines. The percentage cell viability per well was calculated as follows:

$$\% \text{ Inhibition} = 100 - \frac{\text{Corrected mean Absorbance of sample}}{\text{Corrected mean Absorbance of control}} \times 100$$

Three independent assays in triplicate were done, and percentage of growth inhibition was calculated.

2.4. Cell viability assay (Trypan blue dye exclusion assay):

Cells were seeded in the 24 well plates. Sterility was maintained throughout the procedure. The plant extract was added with the concentrations 12.5 µg/ml, 25 µg/ml and 50 µg/ml respectively for 24, 48 and 72 hours to check the effect of doses on the viability of the cells. A cell suspension was made in a fixed volume of cells (2 x 10^6 cells/ ml). At each time interval the cells were trypsinized and removed from the well. Trypsin was removed by centrifugation and cells suspended in the fresh growth media. In the microfuge tubes, a fixed concentration of cell suspension was taken and mixed with an equal volume of trypan blue solution, and was mixed well using a pipette. Then it was transferred to a hemocytometer and observed under a microscope under 10 X magnification. The clear cells were counted as live and blue stained cells were considered as dead. After staining with trypan blue solution, counting was commenced within 5 minutes [16]. Percent viability was calculated using formula:

$$\% \text{ Viability} = \frac{\text{Live cell count}}{\text{Total cellcount}} \times 100$$

Three independent assays in triplicate were done. The viability is depicted as mean percentage here.

2.5. DNA fragmentation Assay (Diphenyl Amine Assay):

DNA fragmentation as an index of percentage apoptosis was measured spectrophotometrically by Diphenyl Amine (DPA) method [17]. As IC_{50} value was obtained after 48 hours incubation, this dose and time interval is considered further. 2 x 10^6 cells/ ml cells were taken and treated with IC_{50} concentration of extract (34.64 µg/ml) for 48 hours. Cells were then harvested by trypsinization in centrifuge tubes and centrifuged. Supernatant was discarded and Phosphate Buffered Saline (PBS) was added to the cell pellets and again centrifuged. Supernatants were taken in separate tubes labeled S and the pellet was suspended in Tris Triton EDTA (TTE) buffer and centrifuged at 20,000 rpm for 10 min at 4° C. The supernatant was transferred in new tubes labeled T. Pellets were obtained and resuspended in TTE and the tubes were labeled B. 25% TCA was added to the tubes and tubes were vortexed vigorously. Tubes were kept overnight at 4° C. Next day tubes were again centrifuged all at 20,000 rpm for 10 minutes at 4° C. The supernatant was discarded and the pellet was hydrolyzed by adding 5% TCA and the tubes were kept at 90° C in a boiling water bath for 15 minutes. Freshly prepared Diphenyl Amine (DPA) was added to the tubes and they were kept overnight at 37° C. Absorbance of the solution was read at 600 nm in an ELISA reader and the percentage of DNA fragmentation was calculated by the formula:

$$\% \text{ of fragmented DNA} = \frac{S+T}{S+T+B} \times 100$$

Three independent assays in triplicate were done and the values are depicted as mean percentage of fragmentation here.

2.6. Agarose Gel Electrophoresis:

DNA fragmentation was assessed by electrophoresis of extracted genomic DNA from HeLa cells as described by Muller *et al.* (1996) [18] with slight modifications. Briefly, 2 × 10^6 cells were incubated with the extract at its IC_{50} concentration at 48 hours and washed with PBS containing 10 mM EDTA. The pellet was lysed in lysis buffer containing NaCl, EDTA, Tris-HCl, Triton X-100, SDS), and incubated at 37° C for 90 min followed by 1 hr incubation with proteinase-K at 50° C. DNA was extracted with phenol: chloroform: isoamyl alcohol for 1 min and centrifuged at 12000 rpm for 3 min. The aqueous phase was further extracted with chloroform and centrifuged. DNA was precipitated from aqueous phase with 3 volumes of chilled alcohol containing 0.3 M sodium acetate. The precipitate was centrifuged at 12000 rpm for 3 min. DNA pellet was washed with 70% alcohol, dried, dissolved in TE buffer and electrophoresed in 1 % gel with Ethidium Bromide. The samples were run on the gel till the bands reached the bottom (as indicated by Bromophenol blue in the loading buffer).

2.7. Acridine orange/ Ethidium Bromide double staining:

25 µl of cell suspension (2.0 × 10^6 cells/ml) after treatment with the IC_{50} concentration of the extract at 48 hours was incubated with 1 µl of Acridine Orange/Ethidium Bromide solution prepared in PBS (pH 7.4) and was mixed gently. Each sample was mixed just prior to microscopy and quantification. Samples were evaluated immediately [19]. 10 µl of cell suspension was placed onto a microscopic slide and covered with a glass coverslip, and examined in a fluorescence microscope using a fluorescein filter and a 10 X objective. At least a total of 300 cells were examined for each experiment. Apoptotic cell percentage was calculated as follows

$$\%\ \text{Apoptotic cell} = \frac{\text{Apoptotic cell count}}{\text{Total cell count}} \times 100$$

Three independent assays were done and the values are depicted as mean percentage of apoptotic cells here.

2.8. Protein Estimation

For protein estimation, cells after dosing with IC_{50} concentration of the extract for 48 hours, were then removed with the help of cell scraper and washed with PBS twice. PBS was discarded and cells were suspended in 500 µl RIPA Buffer and kept on ice for 20 minutes. Cells centrifuged for 15 minutes at 14000 rpm at 4°C. The supernatant was taken into another tube for further analysis [20, 21].

The concentration of proteins was determined by Lowry's method [22] using the Folin-Ciocalteau reagent, with crystalline Bovine Serum Albumin as the standard.

Three independent assays in triplicate were done and Protein levels were expressed as µg / 2 x 10^6 cells.

2.9. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS PAGE):

As a supportive assay, slab gel electrophoresis was performed for protein separation [23]. Cells were treated with the extract at IC_{50} concentration for 48 hours and protein was extracted from the cells by the above mentioned protocol. The protein sample was prepared by mixing sample obtained, with sample buffer containing glycerol, SDS, Bromophenol blue dye, Tris-Cl and β- Mercapto ethanol. Samples were loaded into the wells after the polymerization of gel and were run on the electrophoresis unit. The gels were fixed and then stained 0.25% Coomassie brilliant Blue. After overnight staining, the gels were destained and then photographed and analyzed.

2.10. Statistical analysis

Each parameter was performed in triplicate and the results were expressed as Mean ± Standard Error (S.E.M.). The data was the statistically analyzed by Students't' test and values of $P < 0.05$ were considered statistically significant. In all the parameters, resultant effects of treated cells were compared with untreated control cells.

3. RESULTS

3.1. Proliferation Assay

HeLa cells were grown in 96 well plates for 24, 48 and 72 hours with different concentrations (12.5– 50 µg/ml) of crude plant extract. The formazan crystals were formed, following the reduction of MTT by metabolically active (Viable) cells. The percentage decrease of growth proliferation after treatment with the *Xanthium* extract is given in Figure 1 compared with control as baseline. The IC_{50} value was calculated by plotting the graph of concentration against % inhibition in Microsoft Excel (2003) and was found to be 34.64 µg/ml at 48 hours. There was a significant increase in percentage of inhibition of growth proliferation with increase in the dose and time duration as compared to untreated control cells ($p < 0.001$).

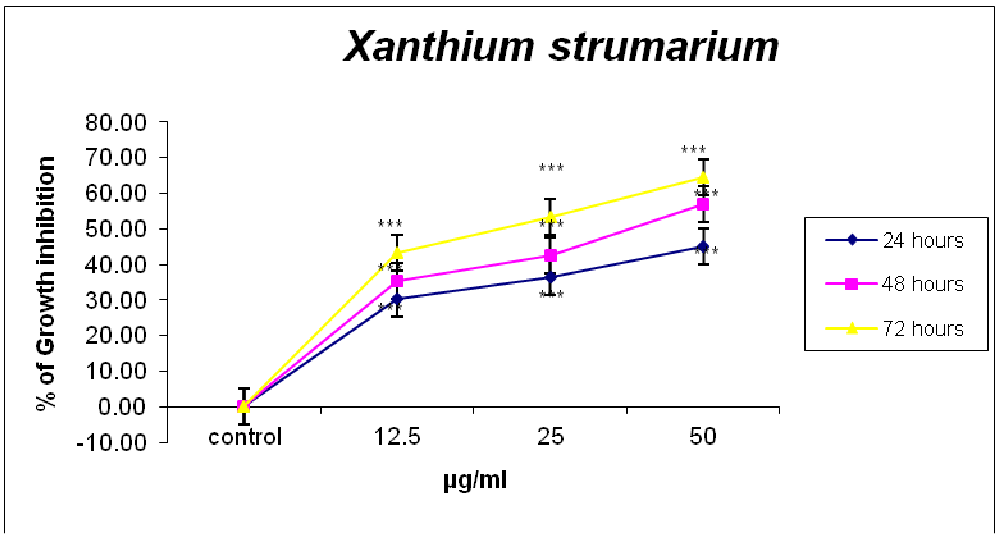

Figure 1. Showing the effect of *Xanthium strumarium* extract on the percentage decrease of growth proliferation in HeLa cells.

*Values are mean ± S.E.M for three individual experiments. Values are % of growth inhibition. ***= p <0.001*

3.2. Viability Assay

The viability assay conducted by Trypan blue dye exclusion method showed that there was a decrease in viability with increase in time duration and concentration in the extract treated HeLa cells as compared to untreated control cells (Figure 2). Highly significant decrease in viability was seen at high dose (50 µg/ml) of the extract ($p < 0.001$).

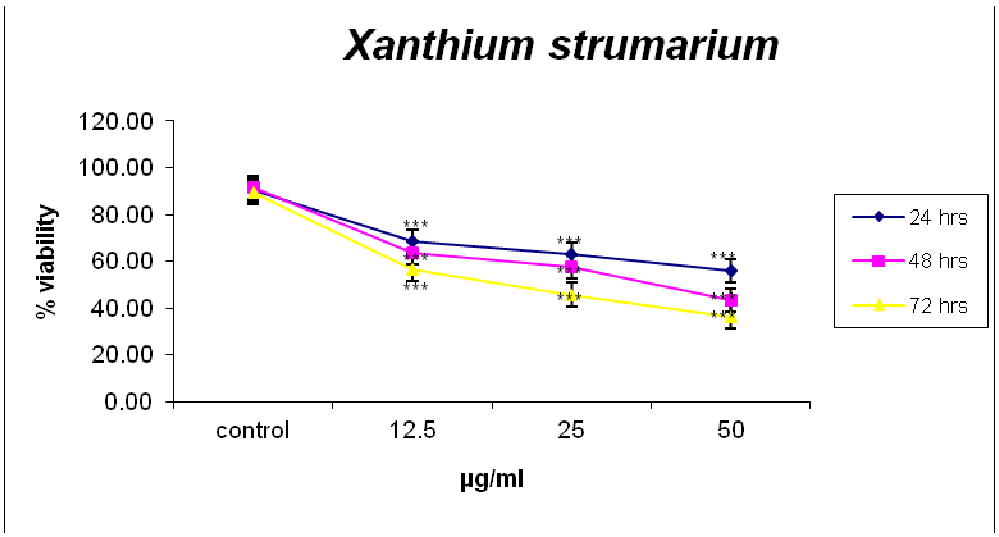

Figure 2. Showing the effect of *Xanthium strumarium* extract on the percentage viability of HeLa cells.

*Values are mean ± S.E.M for three individual experiments. Values are % viability of cells.***= p <0.001*

3.3. DNA fragmentation

DNA fragmentation was determined to measure a late event in cellular apoptosis at 48 hours with the concentration 34.64 µg/ml (IC_{50} dose). Proliferating HeLa (untreated control) cells showed no signs of strand breaks, whereas growth-arrested cells were positive. The extracts induced significant increase in DNA strand breaks (Figure 3).

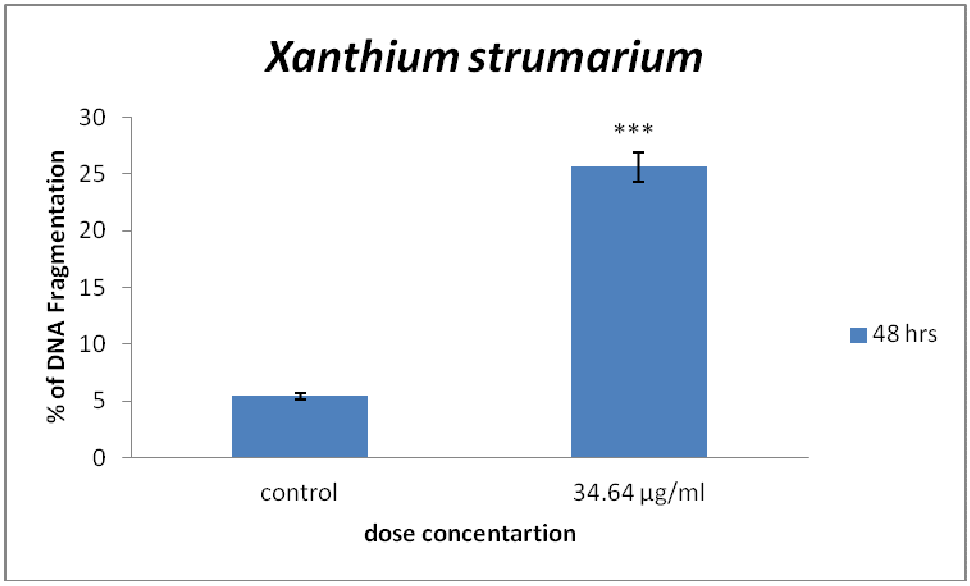

Figure 3. Showing the percentage of DNA fragmentation caused by *Xanthium strumarium* extract in HeLa cells.

*Values are mean ± S.E.M for three individual experiments. Values are % of DNA fragmentation. ***= p <0.001*

3.4. Agarose-gel electrophoresis

Cleavage of chromosomal DNA into oligonucleosomal size fragments is an integral part of apoptosis. Shearing of DNA was observed in treated cells. Untreated cells did not show fragmentation and a single clear band was observed on the agarose gel. When compared with a marker, a band was observed in all the lanes of treated just below 250 bp regions, which suggests that band might be of approximately 180-200 bp fragments (Figure 4).

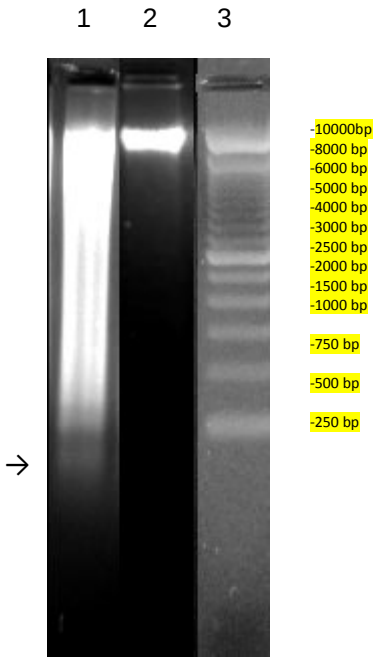

Figure 4. Showing DNA pattern of treated and untreated HeLa cells at 48 hours after dosing, along with marker on Agarose gel electrophoresis.

Lane 1 – Showing effect of Xanthium strumarium at IC_{50} concentrations on HeLa cell DNA, Lane 2 – Untreated control HeLa cell DNA, Lane 3 – DNA Marker

3.5. Acridine Orange/ Ethidium Bromide double staining:

When stained with acridine orange and ethidium bromide dual stain, the live cells took up green colour, and the necrotic or dead cells took orange red colour (Figure 6). The apoptotic cells were seen as

yellowish orange with signs of fragmented nuclei. The results obtained depicted that there was a significant increase (p <0.001) in number of apoptotic cells with increase in time duration (Figure 5).

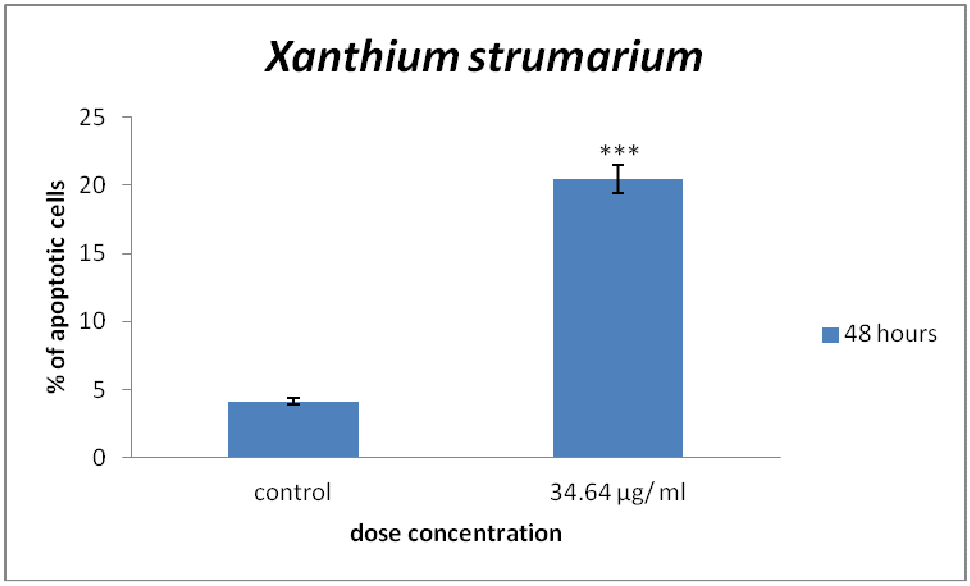

Figure 5. Showing the percentage of apoptotic cells after treatment with *Xanthium strumarium* extract compared with untreated control HeLa cells.

*Values are mean ± S.E.M for three individual experiments. Values are % of Apoptotic cells. ***= p <0.001*

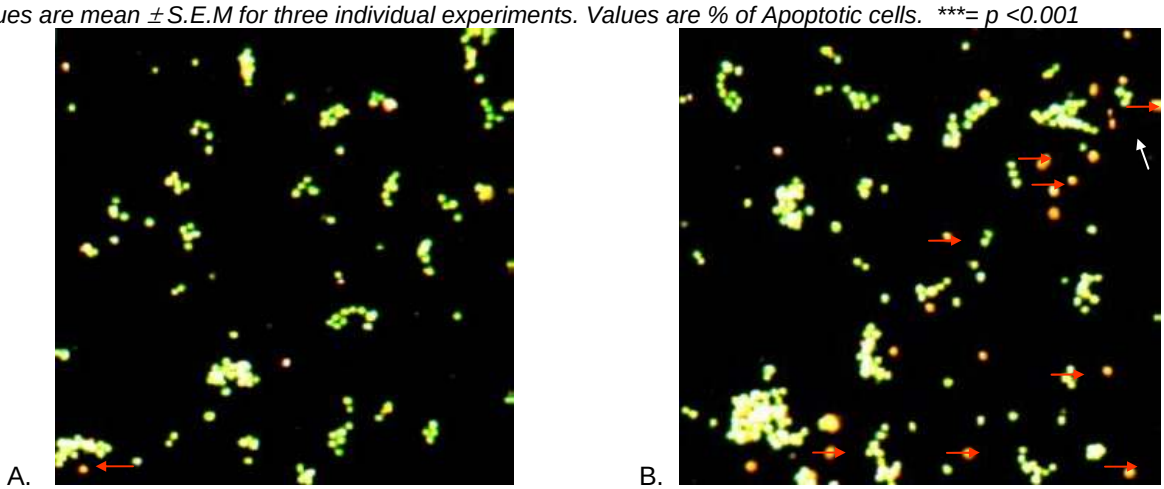

Figure 6. Showing live, apoptotic and necrotic cells with Acridine orange and Ethidium Bromide double staining.

Green cells seen are live cells. Orange arrow shows apoptotic cells and white arrow shows necrotic cell. A. Showing untreated control cells. B. Showing cells treated with 34.64 µg/ml (IC_{50} dose concentration) Xanthium extract concentration for 48 hours.

3.6. Protein estimation

Cells treated with the extract showed a significant decrease in the protein levels as compared to untreated control HeLa cells (Figure 7).

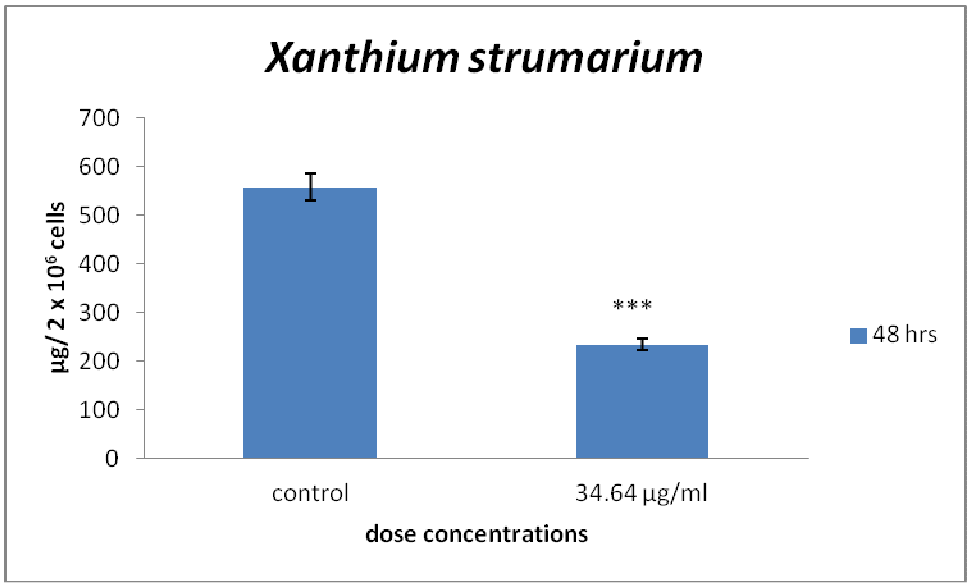

Figure 7. Showing alterations in levels of total proteins after treatment with *Xanthium strumarium* extract compared with untreated control HeLa cells.

*Values are mean ± S.E.M for three individual experiments. Values are µg / 2 x10^6 cells. ***= p <0.001*

3.7. SDS PAGE

The results of SDS PAGE had shown clear dark bands of proteins having an approximate molecular weight of 42 kDa, 51 kDa, 70 kDa and 124 kDa and three faint bands between 124 kDa and 315 kDa in the control HeLa cells. A prominent band of approximate molecular weight of 70 kDa and a faint band of approximate molecular weight 51 kDa were observed in the gel pattern after cells were treated with IC_{50} concentration for 48 hours with the extract of *Xanthium* (Figure 8).

Figure 8. Showing separated protein pattern of treated and untreated HeLa cells at 48 hours after dosing, along with marker on Polyacrylamide gel electrophoresis.

Lane 1 – Showing effect of Xanthium strumarium at IC_{50} concentrations on HeLa cell protein, Lane 2 –Untreated control HeLa cell protein and Lane 3– Protein Marker.

4. DISCUSSION

Plants by virtue of their wide use in traditional medicine and less toxic implications have been drawing the attention of researchers around the world of late. Control of cell cycle progression in cancer cells is considered to be a potentially effective strategy for the control of tumor growth [24-25] as the molecular analysis of human cancers have revealed that cell cycle regulators are frequently mutated in most common malignancies [26-27]. Our *in vitro* data indicated that treatment of HeLa cells with *Xanthium* extract resulted in significant inhibition in proliferation of the HeLa cells. Theoretically, any reduction in the number of metabolically active proliferating cells might mean that the proliferation pathway itself was halted (cell cycle arrest), or that a fraction of the cells went through a death pathway. Therefore, a viability test was necessary to determine which of these two options may play a role in this study. The trypan blue dye exclusion test was used to determine the plant extract-mediated cell death. The unstained cells (viable) and the blue stained (dead) were counted separately, and the percent cell viability was calculated as previously described with the same cell-extract combinations as in the proliferation assay. The percent viability of normally grown cells from the same types was determined for comparison with extract-treated cells and time and concentration dependant reduction in viability was observed. The results obtained could be correlated with MTT assay. These compatible results suggest that reduction in the metabolically active HeLa cells in consequence to *Xanthium strumarium* extract treatment is due to cell death.

One of the goals of anticancer potential of any drug/extract is the induction of apoptosis in cancer cells [28]. Apoptosis or programmed cell death is one of the most important targets for cancer treatment comprising chemotherapy as well as chemoprevention. Deficiencies in the apoptotic pathways may lead to chemoresistance. It is characterized by DNA fragmentation, alteration in membrane symmetry and various morphological changes which lead to activation of cascade of caspases [29]. Chromosomal DNA and mitochondrial dysfunctions play a role on mammalian cell death. The major biochemical dysfunction of chromosome is the presence of an ordered cleavage of the DNA backbone. Mammalian cell death can be induced through chromosomal DNA damage by oxidative stresses such as induced by anticancer drugs and various DNA adduct inducing substances [30]. DNA fragmentation was determined by the diphenylamine method to measure a late event in cellular apoptosis. Proliferating HeLa cells that is control cells showed very less signs of strand breaks, whereas growth-arrested treated cells were positive and showed higher percentage of DNA fragmentation. When the cells were treated with the IC_{50} concentration of the extracts and the DNA was isolated, DNA fragments were observed on agarose gel. Ladder like pattern was observed comprising of large high molecular weight fragments, and when compared with a marker, a band of approximately 180-200 bp was observed giving rise to the possibility of digestion of genomic DNA by an endonuclease into oligonucleosomal size fragments, generating a ladder of small fragments of double-stranded DNA. Once the cells have fragmented their DNA they are committed to die and cannot be rescued by removal of the apoptotic signal. There have been reports of various fractions of *Nigella sativa* (methanolic, n-hexane and chloroform) inducing apoptosis on HeLa cells resulting in DNA fragmentation in a similar fashion [31]. When the cells were stained with Acridine Orange and Ethidium Bromide, it was observed that live cells showed bright green colour whereas, green or orange-red nuclei with condensed chromatin/fragmented DNA indicates apoptotic cells. And bright red necrotic cells were observed. Percentage of apoptotic cells was counted and the results indicate increase in apoptotic cells. Apoptotic cells were seen more in number and only few necrotic cells were observed. Though further confirmation is required, it could be said that the extract might have induced apoptosis, in HeLa cancer cells resulting in DNA Fragmentation [32].

The results from protein estimations showed the significant decrease of protein levels due to effect of the extracts. When the cells were treated with extracts at their IC_{50} concentrations, it was evident that there was a drastic loss of some proteins as seen on the polyacrylamide gel which might have been damaged due to effect of the *Xanthium* extract. A possible reason for the damage of proteins could be the generation of Reactive oxygen species as a result of cell death or as a side effect of any (intrinsic or extrinsic) apoptotic mechanism. Proteins can be severely damaged by oxidative stress-induced carbonylation, S-nitrosylation, glutathionylation, and tyrosine nitration. Among protein oxidative modifications, carbonylation is extremely important, as it occurs at high levels *in vivo* and is often used as a marker of oxidative stress. Protein carbonyls are formed either by a direct oxidative attack on the amino acid side chains of proline, arginine, lysine, and threonine, or by an indirect reaction of the primary amino-group of cysteine, lysine, and histidine with reactive carbonyl compounds (ketoamines, ketoaldehydes,

deoxyosones) or with carbonyl-containing oxidized lipids [33]. Moreover, ROS-induced modifications target several other proteins that are important in cell signaling events, including those involved in cell death or survival, *i.e.*, NFκB, AP-1, H-Ras, MAPK, IP3 kinase, PKC-ε, Ras, p53, HIF-1, ASK-1, Bcl-2, caspases, JNK, and p38 MAPK [33].

Several dietary polyphenols such as resveratrol and caffeic acid have been shown to cause cellular DNA damage through mobilization of endogenous copper ions, possibly chromatin-bound copper, leading to the production of ROS [34-38]. The preferential cytotoxicity toward cancer cells is likely due to the elevated levels of copper, but not iron ions, in cancer tissues and cells [39-40]. As we have mentioned earlier Caffeic acid is one of the active component present in the plant *Xanthium strumarium* [13]. Further studies are required on that line but it can be said that the possible anticancer effect might be due to presence of this component and might be responsible for DNA damage in turn producing ROS which cause protein depletion.

5. CONCLUSION

The findings of our present investigation indicate that the Hydroalcoholic extract of the plant *Xanthium strumarium* showed positive anticarcinogenic effect against HeLa cancer cell line leading to inhibition of proliferation and the loss of viability, which might be the result of induction of apoptosis via signaling of caspase activation mechanism. Though further role of the extract and its exact mechanism of action remains to be explored, it can be suggested that *Xanthium strumarium* extract can be considered as a possible therapeutic agent against human carcinomas.

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