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Preliminary Evaluation of *In vitro* Antiproliferative Activity and Apoptotic Induction by *Tinospora cordifolia* Miers ex Hook F& Thomas on HeLa Human Cervical Cancer cells

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Abstract—*Tinospora cordifolia Miers ex Hook F& Thomas has been used since centuries in Ayurvedic system of medicine for treating various ailments. This medicinal plant was used to explore antiproliferative and apoptotic activity in HeLa cell line. HeLa cells were treated with the hydroalcoholic extract of Tinospora cordifolia in a dose dependant manner and MTT assay was performed at 24, 48 and 72 hours along with the untreated cells to detect the effect of the extract on growth proliferation and IC_{50} value was calculated. DNA of treated cells was isolated and the fragmentation was examined on the Agarose gel along with the DNA of untreated control cells. Percentage of Apoptotic cells was counted. Effect of treatment on HeLa cellular protein level was also determined. The extracts inhibited growth and proliferation of HeLa cells through cell death, which was dose and time dependant. There was induction of DNA Fragmentation and also the increase in number of apoptotic cells suggest that apoptosis was involved in extract induced cell death. A significant loss of cellular proteins was also observed. Although we need further study of chemotherapeutic effect of this plant, the results raise the possibility that the extract of Tinospora cordifolia might be suitable chemotherapeutic agent for treatment of various cancers.*

1. INTRODUCTION

Cervical cancer is the most common cause of cancer death among women in developing countries and the second most common cancer in women worldwide [1]. It is caused by a change in the epithelial cells, which line the wall of the cervix, and the most common risk factor for this type of cancer is the human Papillomavirus (HPV) [1]. Treatment options for cancer majorly include surgery, chemotherapy and radiation therapy which have their own limitations and side effects. Hence, nowadays there is a greater emphasis on treatment with various plant-derived compounds or dietary phytochemicals for cancer control and management [2]. A trend to combine conventional therapy with some form of complementary therapy is growing rapidly [3]. Throughout medical history, plant products have been shown to be valuable sources of novel anticancer drugs. There is a widespread use of herbal medicines depicted even in conventional medical history. Out of 121 prescription drugs in use for cancer treatment, 90 are derived from plant species and 74% of these drugs were discovered by investigating a folklore claim [4-5]. *Tinospora cordifolia* (guduchi) called "amrita" in Sanskrit is a very important drug of the Ayurvedic system. It was used to improve immune system and body resistance against diseases [6]. It is known to be useful in fever, diabetes, dyspepsia, jaundice, urinary problems, chronic diarrhea, dysentery, heart diseases, leprosy, helmenthiasis and rheumatoid arthritis [7-9]. Active components Berberine, palmatine D, choline D, tinosporine,Magnoflorine,tetrahydropalmatine, isocolumbin have been isolated from this plant [10-15]. The focus of our work was to determine the curative role of this plant and the mechanism of action of its extract on HeLa cancer cell line.

2. MATERIALS AND METHODS

2.1 Plant extract

Stem and leaves of *Tinospora* were collected washed and shade dried under ambient temperatureand then powdered.. The powders were then sieved and defatted with Hexane. Hydroalcoholic 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. The yield collected after drying was weighed and kept at 4°C until further use.

The plant extracts for dosing were prepared with the concentrations 1 mg/ml in the 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)[16]. The cells were seeded in the 96 well plate and treated with the extract with the concentrations 50 µg/ml, 25 µg/ml, 12.5 µg/ml respectively for 24, 48 and 72 hours for initial screening and the assay was further carried out. Cell numbers per well were extrapolated from calibration curves for individual cell lines. The percentage cell viability per well was calculated as follows:

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

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

2.4 DNA fragmentation Assay

Agarose Gel Electrophoresis:

DNA fragmentation was assessed by electrophoresis of extracted genomic DNA from HeLa cells as described by Muller *et al.,* 1996 [17] with slight modifications. Briefly, 2 × 10^6 cells were incubated with the extract at the IC_{50} concentration at 72 hours and DNA was extracted, dried, dissolved in 50 µl TE buffer and electrophoresed 1 % gel with Ethidium Bromide.

2.5 Acridine orange/ Ethidium Bromide double staining:

25 µl of cell suspension (2.0 × 10^6 cells/ml) after treatment with the the IC_{50} concentration of the extract at 72 hours with 1 µl of AO/EB solution and was mixed gently and observed under fluorescent microscope [18]. 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.

2.6 Protein Estimation

For protein estimation, cells after dosing with IC_{50} concentration of the extract for 72 hours were 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 [19-20].

The concentration of proteins was determined by Lowry's method [21] 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.

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

As a supportive assay, slab gel electrophoresis was performed for protein separation [22]. The protein sample was prepared by mixing sample obtained (mentioned as above) with sample buffer. 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 with 0.25% Coomassie brilliant Blue. After overnight staining, the gels were destained and then photographed and analyzed.

2.8 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. In all the parameters, resultant effects of treated cells were compared with untreated control cells.

3. RESULTS

3.1 Inhibition of growth proliferation:

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 extracts. 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 selected plant extract is given in Fig. 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 42.10 µg/ml at 72 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. ($p < 0.001$).

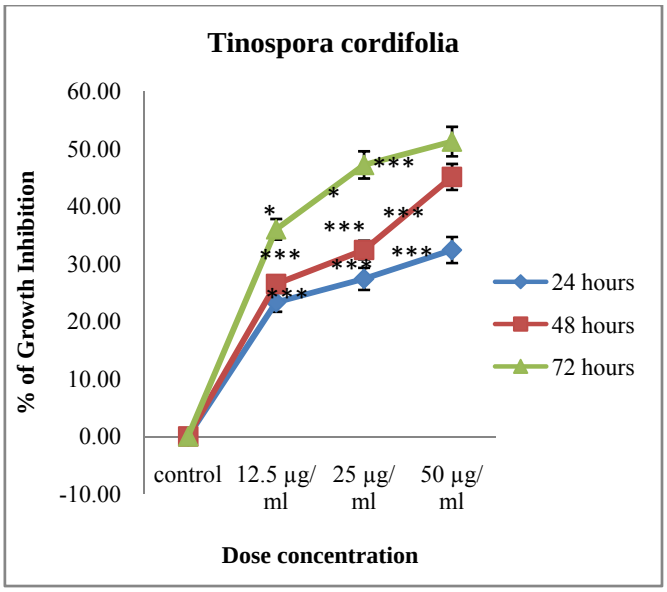

Fig. 1: Showing the effect of *Tinospora cordifolia* extract on the percentage decrease of growth proliferation in HeLa cells. Values are mean ± S.E.M for three individual experiments. Values are % of growth inhibition. ***= p <0.001

3.2 DNA fragmentation

Agarose-gel electrophoresis

Cleavage of chromosomal DNA into oligonucleosomal size fragments is an integral part of apoptosis. Genomic DNA was isolated and analyzed on gel electrophoresis. Agarose-gel electrophoresis of extract treated chromosomal DNA showed DNA fragmentation. Cells were treated with IC_{50} concentrations of the extracts at 72 hours; DNA was extracted and run on the Agarose gel. Heavy base pair fragmentation 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 ladder like appearance is seen in the DNA obtained after treatment with the extract (Fig. 2).

1 2 3

Fig. 2. Showing DNA pattern of treated and untreated HeLa cells at 72 hours after dosing, along with marker on Agarose gel electrophoresis. Lane 1 – Showing effect of *Tinospora cordifolia* at IC_{50} concentrations on HeLa cell DNA, Lane 2 – Untreated control HeLa cell DNA, Lane 3 – DNA Marker

3.3 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. The apoptotic cells were seen as yellowish orange with signs of fragmented nuclei. The results obtained depicted that there was a significant increase in number of apoptotic cells after treatment.

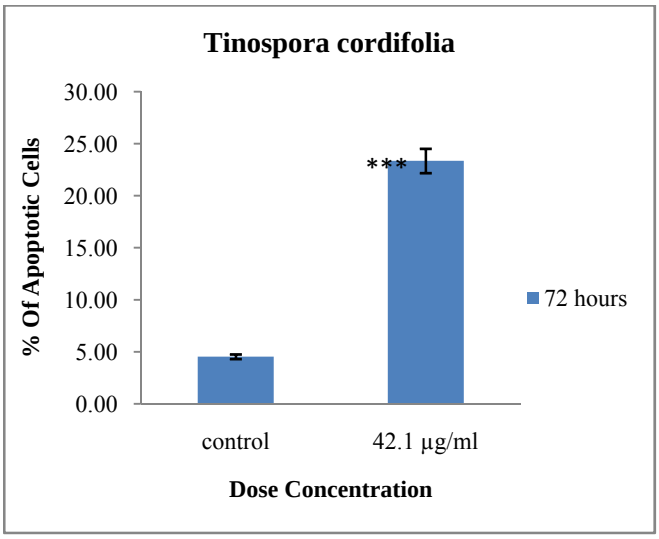

Fig. 3: Showing the percentage of apoptotic cells after treatment with *Tinospora cordifolia* extract compared with untreated control HeLa cells.

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

3.4 Protein estimation

Cells treated with the extract showed a significant decrease in the protein levels as compared to untreated control HeLa cells (Fig. 4).

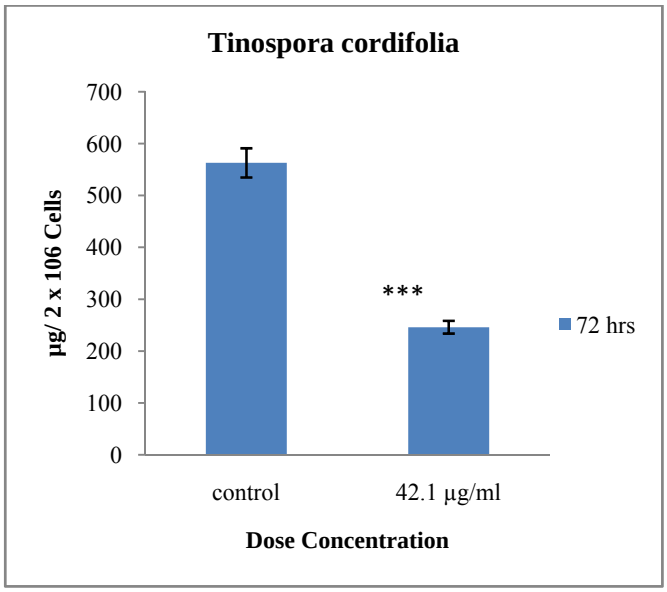

Fig. 4: Showing alterations in levels of total proteins after treatment with *Tinospora cordifolia* extract compared with untreated control HeLa cells.

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

3.5 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 faint bands of approximate molecular weight 51 kDa and 42 kDa were observed in the gel pattern after cells were treated with IC_{50} concentration for 72 hours with the extract of *Tinospora* (Fig. 5).

Fig. 5: Showing separated protein pattern of treated and untreated HeLa cells at 72 hours after dosing, along with marker on Polyacrylamide gel electrophoresis. Lane 1 – Showing effect of *Tinospora cordifolia* 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. *Tinospora cordifolia* (Willd.) Hook. F. and Thoms. is one of the popular herbal formulas throughout the world; yet, evidence-based information is limited. Many studies have reported pharmacological efficacies and benefits of *Tinospora Cordifolia* [23–33], but there is little information on its effect and mechanism of action on cells and cellular protein. Data presented in this report have involved an extensive anticancer assessment of a complex mixture, the whole extract of *T. cordifolia* as part of preliminary studies; the use of various *in vitro* assays was decisive in order to obtain a picture of the anticancer potential of this plant product and to probe further its effects on HeLa cells and propose possible mechanism of action.

Control of cell proliferation in cancer cells is considered to be a potentially effective strategy for the control of tumor growth [34-35] as the molecular analysis of human cancers have revealed that cell cycle regulators are frequently mutated in most common malignancies [36-37]. Our *in vitro* data indicated that treatment of HeLa cells with *Tinospora* 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 (mitotic arrest), or that a fraction of the cells went through a death pathway. Previously, we had worked on the trypan Blue staining for cell viability to verify the influence of the plant extracts in mediating cell death [38]. It was observed in that the cell survival was lowered in a concentration dependant manner. Hence it could be postulated that the decline in cell proliferation, as registered by the MTT assay could be attributed to *Tinospora cordifolia* extract induced cell death as confirmed by the cell viability trypan blue assay.

One of the goals of anticancer potential of any drug/extract is the induction of apoptosis in cancer cells [39]. 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 [40]. Changes in chromosomal DNA play a role on mammalian cell death. The major biochemical damage of chromosome is the presence of an ordered fragmentation of the DNA backbone. Mammalian cell death can be induced through chromosomal DNA damage by oxidative stress such as that induced by anticancer drugs and various DNA adduct inducing substances [41]. It is known that cells with fragmented DNA are susceptible and committed to cell death. DNA of cells treated with IC_{50} concentration of the extracts showed DNA shearing. Also, ladder like pattern was observed comprising of large high molecular weight fragments, and when compared with a marker, 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. Detailed study in this direction is still required, however the results obtained indicate that apoptosis induction might be the possible cause for DNA lysis. Previously, it has been demonstrated by Maliyakkal and co-workers that ethanolic extract of *Tinospora* plants induce DNA Fragmentation in MCF-7 cells seen as a DNA Ladder on agarose gel in similar fashion [42]. When the cells were stained with Acridine Orange and Ethidium Bromide, it was observed that there was higher percentage of cells with condensed chromatin/fragmented DNA which possibly indicate apoptotic cells. These results indicate an increase in apoptotic cells. Apoptotic cells were comparatively higher in number than necrotic cells. Though further confirmation is required, it could be said that the extract might have induced apoptosis, in HeLa cancer cells resulting in DNA Fragmentation [43].

Administration of the *Tinospora cordifolia* extracts also brought about a significant decrease in the total protein content. Further confirmation by PAGE, after treatment with the IC_{50} concentration indicated loss of protein as depicted on the scanned gels by loss of protein bands. Generation of radical oxygen scavenger (ROS) and other radicals above a certain threshold can damage proteins and DNA for certain cancers [44]. DNA damage and fragmentation as detected in this study could possibly result in impaired gene expression,

ROS generation and loss of essential proteins, which in turn could be correlated with low survival of the target cells.

Jagetia (2002), Thippeswamy and Salimath (2007) have shown positive results with the different concentrations of hexane extract of *Tinospora cordifolia* against the Ehrlich ascites tumor (EAT) cells [45-46]. The medicinal values of plants lie in bioactive constituents such as alkaloids, sapopnins, flavonoids, tannins, terpenoids, steroids, glycosides, phenolic compounds [47]. One such component an Isoquinoline alkaloid, Berberine found in the plant has been known to cause a programmed cell death in HepG2, SNU-5, T98G, A431 and human prostate carcinoma cells as evidenced by a reduction in mitochondrial membrane potential [48]. It have been shown to cause apoptotic cellular DNA damage through JNK/p38 MAPK signaling pathway as an effect of the production of ROS [48]. As we have mentioned earlier Berberine is one of the active component present in the plant *Tinospora cordifolia* [10-15] which might be responsible for the anticancer activity of the extract. Further studies are required on that line but it can be said that the possible anticancer effect might be due to presence of one these component and might be responsible for DNA damage in turn producing ROS which causes protein depletion.

5. CONCLUSION

The findings of our present investigation indicate that the Hydroalcoholic extract of the plant *Tinospora cordifolia* 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. Though further role of the extract and its exact mechanism of action remains to be explored, it can be suggested that *Tinospora cordifolia* extract can be considered as a possible therapeutic agent against human carcinomas, since it manifests an *in vitro* inhibition of cell proliferation.

6. ACKNOWLEDGEMENTS

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