



Research Article

CYTOTOXICITY OF CHLOROFORM EXTRACT OF *TODDALIA ASIATICA* L. LEAF

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ABSTRACT

The diverse and magnificent plant kingdom of the world is widely known for its medicinal importance. The potential medicinal properties of plant species have contributed significantly in the development of various herbal therapies for a number of diseases across the globe. The benefits of herbal medicine over allopathic medicine have helped medicinal plants to regain their importance in the field of health and medicine. Cancer is one of the major health problems that have widely affected the world's population. There is a great need to combat this disease with better and more effective medication as compared to existing therapies. *Toddalia asiatica* belongs to Rutaceae, a woody liana, is used traditionally in the treatment of malaria, sprains, cough, fever, neuralgia, epilepsy, dyspepsia and other disease conditions. Extracts of the plant have been reported to have anticancer, anti-HIV, antimicrobial, antifeedant activities. In the present study an attempt has been made to investigate the cytotoxic effect of chloroform extracts of *Toddalia asiatica* leaves on cervical cancer cells. Extracts were studied for their toxicity by trypan blue dye exclusion assay, Cell viability was studied by LDH assay and Apoptosis was measured by DNA fragmentation assay. The study clearly showed the dose dependent cytotoxic effect of extract in HeLa cell line with an CTC₅₀ of 170.00. The LDH leakage was observed in an dose dependent manner with an LDH IU/mg of total protein of 38.92. The dying cells showed characteristics of apoptosis such as, DNA fragmentation at both the concentrations that is at 100 and 200µg/ml. The data in the present study clearly demonstrated cytotoxic effects of extract on human cervical cancer cells.

Key words: Cytotoxicity, *Toddalia asiatica* L. Leaf, HeLa cell line, CTC₅₀ of 170.00.

INTRODUCTION

Cancer is one of the most dangerous, fast propagating with quite high mortality rate disease of present century even in the developed country. The situation is even worse in the under developed country due to lack of knowledge, poverty and non-availability of quality drugs^[1]. Cancer is a disorder developed due to some molecular changes within the cell. It becomes the second major cause of death in the human after cardiovascular disease^[2]. About 7.6 million people died due to cancer in the world during 2007 (American cancer society, Published in May 2007) Hence there is an urgent at present need for developing new approaches and drugs to prevent as well as cure this devastating disease.

India is a rich source of medicinal plants and a number of plant extracts have been used in various systems of medicines such as *Ayurveda*, *Siddha*, *Unani*, etc. to cure various diseases. Only a

few of them have been scientifically explored. Plant derived natural products such as flavonoids, terpenes, alkaloids^[3], etc have received considerable attention in recent years due to their diverse pharmacological properties including cytotoxic and cancer chemo-preventive effects^[4]. *Toddalia asiatica* belongs to family Rutaceae. It grows in forested riparian habitat with high rain fall. It is commonly named as Orange climber.

Two varieties of *Toddalia asiatica*, viz var. *gracilis* and var. *floribunda* are found in the west and southern parts of India. Plants of var. *gracilis* are generally found in denuded slopes. Waste lands and lower hill whereas that of var. *floribunda* found grown only on high altitudes. Several compounds were isolated form T. asiatica Alkaloids like Dihydrochelerythrine and berberin obtained from *T. asiatica* (Syn. *T. aculeata pers.*). (Pakrashi and Bhattacharyya, 1965). Stem yield

compounds like toddalinine, robustine, skimminine, dictamnine, bergapten, luvangetin and isopimpinelline. (deshmukh et al., 1976); Coumarins, fatty acid esters, β -sitosterol, alkaloids and some other compounds (Reis and strobel, 1982). Steam distillates from the leaves of *T. asiatica* shows high antifungal activity against *cladosporium cladosporioides* and cause mortality of the aphid (Bandra et al., 1990). This plant is widely used against stomach problems, malaria, cough, chest pain, sore throat. Though many phytochemical and pharmacological works were carried out on *T. asiatica* no anticancer and DNA protective work is recorded yet and there was no mention in the previous works about the variety on which the works were based. Hence the present investigation is undertaken with a view to bring out anticancer properties of *T. asiatica* leaves extracts.

Taxonomic Description

Domain	: Eukaryota
Kingdom	: Plantae
Subkingdom	: Viridiaeplantae
Phylum	: Tracheophyta
Subphylum	: Spermatophytina
Infraphylum	: Angiospermae
Class	: Magnoliopsida
Subclass	: Rosidae
Superorder	: Rutanae
Order	: Rurales
Suborder	: Rutineae
Family	: Rutaceae
Subfamily	: Toddaliodeae
Genus	: Toddalia
Species	: Toddalia asiatica



Figure 1: *Toddalia asiatica* L.

Materials and Methods

Identification of Plants

Plants species were collected from the forest with the help of local elder tribal head and are identified with local flora of Tirupati. The vouchers specimens were deposited in the Herbarium of Botany department, S.V. University, Tirupati, Andhra Pradesh.

Preparation of the Extract

5 gms of fresh leaves of *Toddalia asiatica* were weighed using a weighing balance and washed with distilled water and grinded in mortar and pestle using 5 ml of chloroform. Then they are poured in petri plates and kept in an incubator at 37°C for 1 day. Air dried extracts were stored at 20°C until analysis.

Chemicals

Trypan blue, Fetal Bovine serum (FBS), Phosphate Buffered Saline (PBS), Dulbecco's Modified Eagle's Medium (DMEM), Modified Eagle's Medium (MEM) and TPVG were obtained from Sigma Aldrich Co, St Louis, USA. EDTA, Glucose and antibiotics from Hi-Media Laboratories Ltd., Mumbai. Dimethyl Sulfoxide (DMSO) and Propanol from E. Merck Ltd., Mumbai, India.

Cell line and Culture medium

HeLa (Human cervix carcinoma) cell lines were procured from National Centre for Cell Sciences (NCCS), Pune, India. Stock cells were cultured in DMEM and MEM respectively, supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/ml), streptomycin (100 μ g/ml) and amphotericin B (5 μ g/ml) in humidified atmosphere of 5% CO₂ at 37°C until confluent. The cells were dissociated with TPVG solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). The stock cultures were grown in 25 cm² culture flasks (Tarsons India Pvt. Ltd., Kolkata, India).

Preparation of Test Solutions

For cytotoxicity studies, each weighed test drugs were separately dissolved in distilled DMSO and volume was made up with DMEM/MEM supplemented with 2% inactivated FBS to obtain a stock solution of 1 mg/ml concentration and sterilized by filtration. Serial two fold dilutions were prepared from this for carrying out cytotoxic studies.

Determination of cell viability by trypan blue dye exclusion technique

The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0×10^5 cells/ml using DMEM/MEM containing 10% FBS. To each of 40mm petri dish, 1 ml of the diluted cell suspension (approximately 100,000 cells) was added. After 24 h, when a partial monolayer was formed, the supernatant was removed, washed the monolayer once with medium and 1 ml of different test concentrations of test drug was added on to the partial monolayer in culture dishes. The dishes were then incubated at 37°C for 3 days in 5% CO₂ atmosphere, and microscopic examination was carried out and observations were noted every 24 h interval. After 72 h, the drug solutions in the wells were removed and cells were trypsinized. The cells were suspended in PBS and centrifuged to separate cell pellet, resuspended in 1 ml of fresh medium and performed dye exclusion test i.e. equal quantity of the drug treated cells and trypan blue (0.4%) were mixed and left for a minute. It was then loaded in a haemocytometer and viable and non-viable count was recorded within two minutes. The percentage growth inhibition was calculated and CTC₅₀ value is generated from the dose-response curves for each cell line.

$$\% \text{ Growth Inhibition} = 100 \left(\frac{\text{Total Cells} - \text{Dead Cells}}{\text{Total Cells}} \right) \times 100$$

DNA fragmentation studies

HeLa cells (3×10^6 /ml) were seeded into 60mm Petri dishes and incubated at 37°C with 5% CO₂ atmosphere for 24 h. The cells were washed with medium and were treated with extract,

standard drug and incubated at 37°C, 5% CO₂ for 24 hrs. At the incubation time ended, the chromosomal DNA of cancer cells was prepared with G Biosciences, USA apoptotic DNA ladder kit. The recovered DNA was loaded onto 2% agarose gel electrophoresis and run 50 V/cm for 3 hrs. The gel was visualized under UV transilluminator and photographed.

LDH leakage assay

The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0×10^5 cells/ml using DMEM medium containing 10% FBS. To each well of the 96 well microtitre plate, 0.1 ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 hrs, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with medium and 100 µl of different test concentrations of test drugs were added on to the partial monolayer in microtitre plates. The plates were then incubated at 37°C in 5% CO₂ atmosphere. After 24 hrs, the drug solutions in the wells were pooled separately and LDH levels were estimated as per manufacture instructions (ERBA diagnostics).

RESULTS

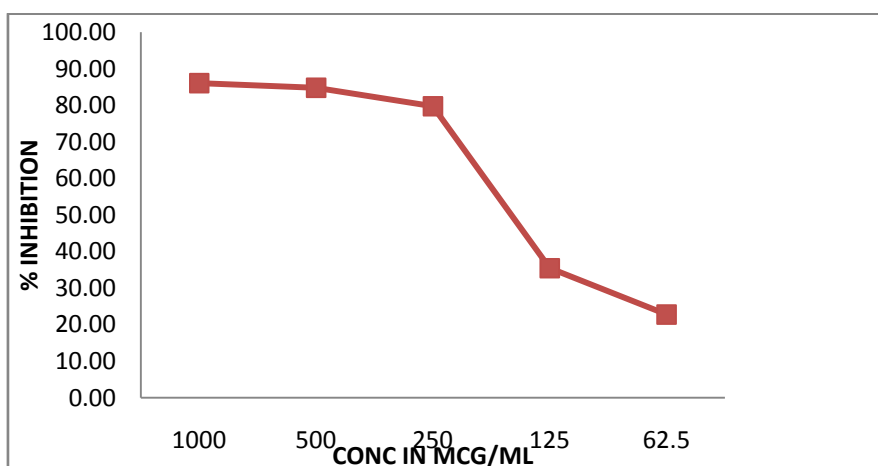
The chloroform extract was studied for cell viability by using Trypan blue exclusion assay and Lactate dehydrogenase (LDH) leakage assay were performed in HeLa cell line. The trypan blue exclusion assay was carried out with the concentrations ranging from 6.25 to 1000 % (v/v). percentage of cell death was observed to be dose dependent. The CTC₅₀ value in % (v/v) is 170.00 as shown in table I.

Table 1: Cytotoxic studies on HeLa cell line by Trypan blue dye exclusion assay

S.NO	Test Drug	Test conc. In % (v/v)	% Cell death	CTC 50 In % (v/v)
	<i>T.asiatica</i>	1000	86.08	170.00
		500	84.81	
		250	79.75	
		125	35.44	
		62.5	22.78	

Graphical representation

Cytotoxic effect of the extracts on HeLa Cell line



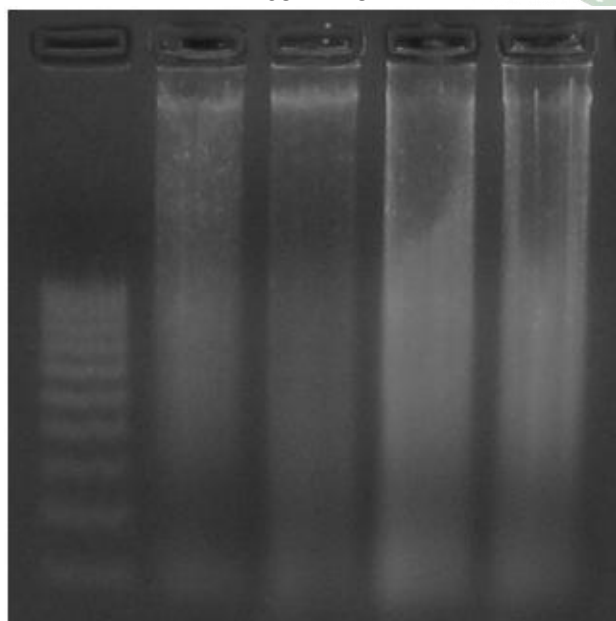
Whereas the activity of LDH leakage was significantly increased. The increased LDH activity was in a dose dependent manner and more LDH leakage was observed with higher concentration of extract that is at 150 µg/ml the LDH IU/mg of total protein was 38.92 as shown in table II.

Table 2: LDH levels in treated test drugs in HeLa cell line

S.No	Name of Test sample	Test Conc. (µg/ml)	LDH IU/mg of total protein
1	<i>T.asiatica</i>	150	36.18
		100	25.18
		50	18.5
2	Doxorubicin	1.5	67.8
3	Cell control	-	14.47

DNA fragmentation was performed by using the DNA ladder assay, the DNA isolated from HeLa cells treated with 100 and 200 µg/ml of the extract showed fragmentation as shown in fig 1

Figure 2: DNA fragmentation studies of HeLa cell line



Lane A: DNA ladder
Lane B: HeLa cells (Untreated)
Lane C: Standard (5µg/ml)
Lane D: HeLa cells treated with extract (200 µg/ml)

Lane E: HeLa cells treated with extract (100 µg/ml)

DISCUSSION

This study was undertaken to scientifically prove the traditional claim of extract of *T.asiatica* possessing anticancer activity. An attempt was made to determine the cytotoxicity of extracts by Trypan Blue exclusion, LDH assay and DNA fragmentation. Trypan Blue exclusion method was very simple & precise. Live cells or tissues with intact cell membrane are not coloured. Since cells are very selective in the compounds that pass through the membrane, in a viable cell trypan blue is not absorbed; however, it traverses the membrane in a dead cell. Hence, dead cells are shown as a distinctive blue colour under a microscope. Since live cells are excluded from staining, this staining method is also described as a dye exclusion method. *T.asiatica* showed an CTC₅₀ of 170.00. From this assay it is clear that *T.asiatica* extracts showed moderate toxicity.

Measurement of LDH activity is another indicator of cell viability through evaluation of the cell membrane permeability. The enzyme activity

is measured externally, as it leaks from dead cells which lose their membrane integrity. LDH leakage detection is based on the loss of NADH due to its oxidation to NAD⁺, resulting in the conversion of pyruvate to lactate. Untreated cells should retain LDH in their cells and have minimal loss over the time of the assay.

Table 2 show the leakage of LDH in the culture media after treatment with extracts in increasing concentrations respectively. Exposure of HeLa cells to *T.asiatica* showed the increase of LDH leakage from 15.41 to 38.92 LDH IU/mg of total protein content. When HeLa cells were treated with doxorubicin, LDH was released in a time and concentration dependent manner. Besides, HeLa cells were more sensitive to doxorubicin and less sensitive to extracts

Apoptotic DNA fragmentation is a key feature of apoptosis, a type of programmed cell death. Apoptosis is characterized by the activation of endogenous endonucleases with subsequent cleavage of chromatin DNA into internucleosomal fragments of roughly 180 basepairs (bp) and multiples thereof (360, 540 etc.). This effect can be used to detect apoptosis, for example via the DNA laddering assay. Plant extract showed the DNA fragmentation in both concentrations 100 and 200 µg/ml.

CONCLUSION

Therefore, it can be concluded that the plant extract possess potent antitumor activity on HeLa cancer cells. Further studies can be undertaken for the isolation and characterization of active chemical constituent from these plants which may act as a lead compound for the development of potential anticancer drugs.

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