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Research Article

CYTOTOXICITY OF CHLOROFORM EXTRACT OF *WALTHERIA INDICA* L. LEAF

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ABSTRACT

India has the richest bio-resource of medicinal plants that have long life history of application in the treatment of diseases from the ancient times. The drugs that are obtained from the medicinal plants have been well favored by the people because of its treatment efficacy which is less when compared to the modern drugs but causes lesser side effects. This advantage lead the research field of pharmacy to discovery of the active constituents that are responsible for causing the pharmacological action on human body. The active constituents from the medicinal plants are more effective and do not cause any side effects. This study has been made to investigate the cytotoxic effect of chloroform extracts of *Waltheria indica* leaves on cervical cancer cells. Extracts were studied for their toxicity by MTT assay, Trypan blue dye exclusion assay, cell viability was studied by LDH assay and Apoptosis was measured by DNA fragmentation assay. The extract screened for phytochemical analysis was found to contain bioactive compounds like carbohydrates, glycosides, saponins, phenolic compound and triterpenoids were present..The cytotoxicity study clearly showed the dose dependent cytotoxic effect of extract in HeLa cell line with an CTC_{50} of 103.33±5.77 for MTT assay and 90.00 for Trypan blue dye exclusion assay. The LDH leakage was observed in a dose dependent manner with an LDH IU/mg of total protein of 36.18. The dying cells showed characteristics of apoptosis such as, DNA fragmentation. The data in the present study clearly demonstrated cytotoxic effects of extracts on human cervical cancer cells.

KEYWORDS: MTT assay, Trypan blue dye exclusion assay, LDH assay, Apoptosis, DNA fragmentation, HeLa cell line.

INTRODUCTION

Carcinogenesis is a multistep process it includes initiation, promotion and progression stages and various require agents promote the development of cancer in each stages [1,2]. Cancer is a cellular disease and mainly caused by the misbalance of the normal cellular growth maturation and multiplication. Main feature of the cancer is chromosomal change and disease primarily caused by alteration in the genome of the affected cells. Cancer is one of the leading causes of death around the world. Herbal drugs have been used since ancient times as medicines for the treatment of a cancer. Medicinal plants have played a key role in world health. An increasing number of research papers and reviews clearly indicate that medicinal plants exhibit a variety of therapeutic properties. [Ahmad et al.1998;Datta et al.1998; Abo et al.2000; Neto et al.2002] and provide health security to rural people in primary health care.

W. indica is also known as velvet leaf, marsh-mallow, monkey bush, boater bush, leather coat, buff coat, and many other names (Burkill,2000) belong to the family *Sterculiaceae*. It is found throughout the tropics and warmer subtropics of India. The plant has been used as an infusion or decoction where febrifugal, purgative, emollient, tonic, analgesic and astringent action is sought (Burkill, 2000). In some places it is used to make herb tea. Stems are used as a chew stick; extracts of the plant are used for treatment of cough and curing female sterility. The root is chewed to relieve sore throat as well a treatment of gonorrhea and leprosy in humans. Stems are used as a chew stick while its extracts are used as an eye bath and a remedy for hemoptysis, treatment of cough and a cure for female sterility (Wagner et al., 1990).

Kingdom - Plantae
Subkingdom - Tracheobionta
Super division - Spermatophyta
Division - Magnoliophyta

Class - Magnoliopsida
Subclass - Dilleniidae
Order - Malvales
Family - Sterculiaceae
Genus - Waltheria L.
Species - *Waltheria indica* L.

The plant (part not specified) is antisiphylitic and febrifuge [1]. A decoction of various plant parts is taken as a treatment for fever and syphilis. It is applied externally on skin eruptions and wounds. A decoction of the leafy stems is taken to relieve fevers, coughs, colds, bladder ailments, vaginal infections, hypertension, ulcers and as a remedy for haemoptysis [De Filipps et.al]. A decoction of the root is given as an antidiarrhoeal and general tonic to children [1]. It is also used as a cough medicine and for healing wounds. A general screening of the plant revealed the presence of some general flavonoids and caffeic acid. By this medicinal efficacy this plant material is subjected to evaluate the anticancer potential and DNA protective activity by using *invitro* techniques. The present study made to investigate the cytotoxic effect of chloroform extracts of *W. indica* leaves on cervical cancer cells. Extracts were studied for their toxicity by MTT assay, Trypan blue dye exclusion assay, cell viability was studied by LDH assay and Apoptosis was measured by DNA fragmentation assay.

Materials and Methods

Identification of Plants

Plants species was collected from the forest with the help of local elder tribal head and are identified with local floras of Tirupati. The vouchers specimens were deposited in the Herbarium of Botany department, S.V.University, Tirupati. Present work has been carried out in Department of Botany, S.V.University, Tirupati.

Preparation of the Extract

5 gms of fresh leaves of *Waltheria indica* 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), 3-(4,5–dimethyl thiazol–2–yl)–5–diphenyl tetrazolium bromide (MTT), 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 μg/ml) and amphotericin B (5 μg/ml) in humidified atmosphere of 5% CO_2 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 cm^2 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.

Preliminary Phytochemical Screening

Phytochemical screening for alkaloids, flavanoides, quinines, glycosides, terpenoids and saponins were analyzed using the powdered chloroform leaves extract using the method of Rosenthaler, L., 1930 and Kokate, 2001.

Determination of cell viability by MTT Assay

The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0 x 10^5 cells/ml using DMEM 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 h, 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 for 3 days in 5% CO_2 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 discarded and 50 μl of MTT in PBS was added to each well. The plates were gently shaken and incubated for 3 h at 37° C in 5% CO_2 atmosphere. The supernatant was removed and 100 μl of propanol was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 540 nm. The percentage growth inhibition was calculated using the following formula and concentration of test drug needed to inhibit cell growth by 50% (CTC_{50}) values is generated from the dose-response curves for each cell line.

$$\% \text{ of inhibition} = 100 - \frac{\text{Mean OD of individual test group}}{\text{Mean OD of Control group}} \times 100$$

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 x 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% CO_2 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 CTC_{50} value is generated from the dose-response curves for each cell line.

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

DNA fragmentation studies

HeLa cells (3 x 10^6 /ml) were seeded into 60mm Petri dishes and incubated at 37°C with 5% CO_2 atmosphere for 24 h. The cells were washed with medium and were treated with extract, standard drug and incubated at 37ºC, 5% CO_2 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 x 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 h, 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% CO_2 atmosphere. After 24 h, the drug solutions in the wells were pooled separately and LDH levels were estimated as per manufacture instructions (ERBA diagnostics).

RESULTS: The preliminary phytochemical screening of *W.indica* has revealed the presence of carbohydrates, glycosides, saponins, phenolic compound and triterpenoids were present. Alkaloids were completely absent as given in Table - I.The chloroform extract was studied for cytotoxicity by using MTT assay, cell viability by using Trypan blue exclusion assay, Lactate dehydrogenase (LDH) leakage assay were performed on HeLa cell line. MTT assay showed the dose dependent response with a CTC_{50} value of 103.33±5.77 as shown in table II.Morphological observations have been carried out as shown in figure I. 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 CTC_{50} value in % (v/v) is 90.00 for *W.indica* as shown in table III ,where as 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 36.14 for *W.indica* as shown in table IV. Furthermore when compared with extract Doxorubicin (positive control) showed higher LDH activity toward cell lines. 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 extracts showed fragmentation as shown in fig II.

Table I: Phytochemical Profile

	TEST	CHLOROFORM EXTRACT
1.	Carbohydrates	+
2.	Glycosides	+
3.	Saponins	+
4.	Alkaloids	
	a. Mayers Test	-
	b. Dragendrodrojj's Test	-
5.	Flavonoids	-
6.	Phenolics & Tannis	
	a. Ferric Chloride Test	-
	b. Test for Tannins	-
7.	Phytosterols & Triterpenoids	
	a. Leiberman – Bucharat	+
	b. Salkowaski Test	+
8.	Oils & Fats	-

Table II: Cytotoxic studies on HeLa cell line by MTT assay

S. No	Name of Test sample	Test Conc. (µg/ml) %	Cytotoxicity (µg/ml)	CTC 50
1.	*W .indica*	1000 500 250 125 62.5	85.77±0.6 85.52±0.3 67.85±0.9 52.39±1.3 45.94±4.9	103.33±5.77

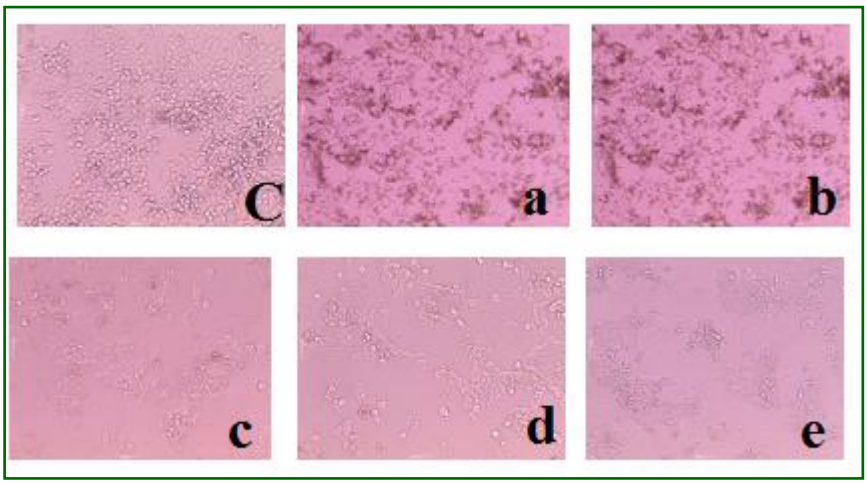
Table III: Cytotoxic studies on HeLa cell line by Trypan blue dye exclusion assay:

S.No	Test Drug	Test concn. in % (v/v)	% Cell death	CTC 50 In % (v/v)
1.	*W.indica*	1000 500 250 125 62.5	86.89 86.89 70.49 54.10 44.26	90.00

Table IV: 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	***W.indica***	150 100 50	36.18 25.18 18.5

Fig I :Morphological observation of HeLa cells by MTT assay



(a)62.5µg/ml (b)125µg/ml (c)250µg/ml (d)500µg/ml (e)1000µg/ml

DISCUSSION

Research on biologically active compounds from essential oils has proved them to be potent anti-bacterial, antifungal and anti-oxidant agents (Lampronti *et al*., 2006; Albuquerque *et al*., 2007; Ao *et al*., 2008; Baik *et al*., 2008; Bakkali *et al*., 2008). Cellular proliferation depends on the rates of cell division and death and, thus, many anticancer drugs have been used to prevent cancer cell division in order to inhibit cancer cell proliferation. In vitro cytotoxicity assays can be used to predict human toxicity and for the general screening of chemicals (Clemedson and Ekwall, 1999; Scheers *et al*., 2001). It has been previously reported that different cytotoxicity assays can give different results depending on the test agent used and the cytotoxicity assay employed (Weyermann *et al*., 2005). The results obtained from the cytotoxicity assays indicate that inhibition of HeLa was gradually increased by the addition of extract in MTT and LDH assays. It reveals that HeLa cell line is susceptible to some of the component in extract in extract. Although, the LDH leakage assay is based on the release of the enzyme into the culture medium after cell membrane damage whereas the Trypan blue dye exclusion based on live cells or tissues with intact cell membrane are not coloured both the cytotoxicity assays, employed to assess extract toxicity *in vitro*, showed the similar results. Apoptosis is a physiological process of cell elimination, and DNA fragmentation is one of the hallmarks of cell apoptosis. The results showed the apoptosis proportion of cells was increased by treatment of carvacrol in both the cervical cancer cell lines.

CONCLUSION

From the present findings, it can be concluded that the studied extract of *W.indica* showed toxicity against HeLa cell line in all the cytotoxic assays studied. The results showed moderate toxicity toward

cancerous cell line.Hence the extracts need to be thoroughly studied using animal models.

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