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In vitro anticancer activities and cytotoxic studies of the leaf extracts of *Ichnocarpus frutescens* (L.) WT. aiton against HeLa cell line

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Abstract
Ichnocarpus frutescens (L.) W.T. Aiton is an important medicinal plant of the family apocynaceae used to cure various ailments. Leaf extracts of *I. frutescens* was prepared by using different solvents like petroleum ether, chloroform, ethyl acetate, ethanol and water to screen their anticancerous activity against HeLa (Human cervical cancer cells) cell lines. Chloroform leaf extract showed significant cytotoxicity in MTT assay against HeLa cell line with IC_{50} value 188.6µg/ml. It was further tested for their apoptotic activity, caspase-3 expression and Cell Cycle analysis using AnnexinV-PI staining, caspase-3 antibody staining and Propidium Iodide Staining respectively. The results of the present study indicated that *I. frutescens* have anticancerous effect on the HeLa cancer cell line with the induction of apoptosis, caspase-3 enzyme expression and cell cycle arrest. This plant may be recommended for the formulation of new therapeutic drugs to cure human cervical cancer.

Keywords: *Ichnocarpus frutescens*, cytotoxicity, MTT, HeLa cell lines

Introduction
Cancer is a group of diseases includes abnormal cell growth with the possible to invade or proliferate to other parts of the body. There are several types of cancer in human being; among these the cervical cancer is the fourth most common cancer in women. In 2018, approximately 3,11,000 women died from cervical cancer [3]. *I. frutescens* is commonly called Krishna Sariva, has been traditionally used to cure different types of cancer because of its potent medicinal properties. The whole parts (root, stem, leaves and flower) of *I. frutescence* used for treating various malady like Rheumatism, night blindness, measles, dysentery, fever, asthma, cough, bronchitis, bone fracture, cholera, constipation, ulcer, vomiting tonic, febrifuge, leukoderma reported by Adhikari, *et al.*, (2010) [1], loss of sensation, hemiplegia, headaches, demulcent, syphilis, diabetes mellitus, excretion of stone in the bladder, wound between fingers, diaphoretic, diuretic, dyspepsia, and purification of blood[12]. The roots of *I. frutescens* are used in medicine as a substitute of Indian sarsaparilla (*Hemidesmus indicus*) [11]. The leaf latex of this plant used to treat skin infections by Siddis of Uttara Kannada district, Karnataka, India [6]. Latex of the plant is used to treat tumors which help full in reduce the pain and retard the growth used by local populations in five sacred groves of Cuddalore district in Tamilnadu, India [2]. *I. frutescens* roots were used to cure stomach cancer in combination with the roots of *Cissampelos pareira*, *Bauhinia vahlii* and *Ardisia solanace* by tribal's of Chotanagpur and Santhal Parganas of Bihar, India [12]. 4-vinyl cyclohexane induced ovarian cancer in Swiss albino mice were treated with ethanolic extracts of *I. frutescens* proved anticancer activity investigated by Thangarajan *et al.*, 2013 [16]. *I. frutescence* has also been used to cure risk of many diseases including cancer. Hence the present study is aimed at analysing the *in-vitro* Anticancerous activity of *I. frutescens* against Hela cell line which cause Human cervical cancer.

Materials and Methods
Collection of plant material
Plant materials were collected from Doresani Palaya Forest Campus, Bengaluru. The plant was identified and authenticated by Botanical Survey of India, Western Regional Centre, Pune, India (plant identification letter No.BSI/WRC/IDEN, CER/2018/H_2/56, voucher specimen number: 136265).

Preparation of plant extracts
The leaves of the *I. frutescens* collected and immediately washed with tap water, they were air dried at 25ºC for 5 days under shade. The dried leaves were powdered well using grinder. This powdered material was stored in an air tight container**.** 10g leaves powdered of *I. frutescens* is weighed and extracted individually using 150ml of 5 different solvents like petroleum ether, chloroform, ethyl acetate, ethanol and water (least polarity to most polarity) for 72 h in soxhlet apparatus. All the five concentrated crude extracts were collected and allowed for drying and stored at 4º C for further studies.

Cell lines and chemical reagents
HeLa-Human Cervical Cancer Cell line, cell culture medium: DMEM- High Glucose (Himedia), Fetal Bovine Serum (Himedia), MTT Reagent (Himedia), Camptothecin (Sigma), DMSO (Sigma), D-PBS (Himedia), Tripsin (Himedia), Annexin V (BD Biosciences), propidium iodide stain (BD Biosciences), BD Cytofix / Cytoperm solution (Himedia), Anti–Caspase-3 antibody (BD Biosciences), PI/RNase A solution (Himedia), Sodium azide (Himedia).

Cell culture
Hela cell line (Human cervical cancer) was procured from NCCS, Pune and were grown and maintained in (Dulbecco's Modified Eagle's medium (DMEM) and supplemented with high glucose and an antibiotic (50U/mL

of Benzyl penicillin, 50µg/mL of Streptomycin and 50µg/mL of Amphotericin-B) and 5% fetal bovine serum (FBS) (Growth medium) in a humidified 5% CO_2 incubator at 37º C, until they reached confluency. Later, they were washed with PBS and treated with trypsin for cell counting and seeding.

Cytotoxicity (MTT) assay

To determine the anticancer activity of leaf extracts of *I. frutescens* on Hela cancer cell line was performed by MTT 3-(4, 5-Dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide (MTT) assay [13]. The cultured cells were collected when they reach about 70-80% confluency. Further the 200µl cells were seeded on to 96 well plates at required density (2×10^4 cells per well) and incubated in 5% CO_2 incubator. Then the cells were allowed to grow for about 12 hours. 1 mg of different extracts of IF were dissolved in 50µL of DMSO (dimethyl sulphoxide) separately, was made up to 1000µL of this solution of culture media. Later, the cells were treated with various concentrations of test samples (10, 50,100,200 and 400µM) and incubated for 24 hrs. Subsequently remove spent media and add 100µL MTT reagent to each well and incubated further for 3 – 4 hrs. After the incubation period, the MTT reagent was discarded by pipetting out without disturbing the cells and 100µL DMSO was added to solubilize the formazan (MTT reduced to formazan by living cells). Cytotoxicity can also be compared with the positive control using standard drug (Camptothecin 25µM) and negative control as DMSO against the HeLa cell lines.

Absorbance was recorded with ELISA reader at 570 nm. The IC_{50} value was determined by using Graphpad prism version 6.0.

Calculating % of inhibition

$$\% \text{ Inhibition} = [\text{OD of Control} - \text{OD of sample} / \text{OD of control}]\times 100$$

The extract which showed lowest IC50 value of MTT assay were considered for further analysis.

Cell cycle analysis by Pi staining

Dissemination of Hela cells was investigated in different phases of the cell cycle, cells were seeded at the density of 2 x 10^5 cells/2 ml in a 6-well plate and incubated in a CO_2 incubator for 24 hours at 37°C for cell adherence. Subsequently, the cells were treated at a lowest concentration corresponding to their IC_{50} value of MTT assay and incubated for 24 hours. Then, the cells were trypsinized and washed with ice cold PBS, followed by treatment with 70% ethanol and allowed to fix for at least 30 minutes on -20$^{\circ}C$. Later, the tubes were centrifuged for 5 minutes, the supernatant was decanted and the cells were washed with PBS twice and incubated with 400µl of PI (propidium iodide) solution for 5 to 10 min at room temperature. Cell cycle analysis of samples was estimated by flow cytometry (BD FACS Calibur) in PI/RNase A solution [5].

Apoptosis by annexin v–PI staining

HeLa cells were cultured at the density of 3 x 10^5 cells/2 ml in a 6-well plate and incubate in a CO_2 incubator at 37°C for 24 hours. Treated the cells with lowest IC_{50} value of the MTT assay and cells were incubated. The media was removed from all the wells and the cells were washed with PBS. Further, the PBS was removed and 180µl of trypsin-EDTA solution was added and incubated at 37°C for 3-4 minutes. Later, 2 ml of culture media were added into each well and harvested the cells directly into 12x75 mm polystyrene tubes. The tubes were centrifuged at 300× g at 25°C for five minutes and the supernatant was decanted carefully. Cells were washed twice with PBS and decanted the PBS completely. Transfer 100µl of the solution (1x105 cells) to a 5 ml culture tube containing 5µl of FITC Annexin V. The contents were gently vortexed and incubated at room temperature (25ºC) for 15 min in dark. Later the cells were treated with 5µl of PI and 400µl of 1X Binding Buffer and vortexed gently again. Apoptotic cells were analysed by flow cytometry (BD Biosciences, San Jose, CA, USA) immediately after addition of PI [7].

Caspase 3 expression Studies by flow cytometry

Anticancer property of this plant was anlayzed by caspase 3 expression using Caspase 3 antibody which is proapoptotic marker or biomarker was treated the HeLa cells. The cells were cultured at the density of 3x 10^5 cells/2 ml in a 6-well plate and incubated in a CO_2 incubator at 37°C for 24 hours to allowed for the cell adherence. Later, cells were treated with test sample at a concentration equivalent to their lowest IC_{50} value of cytotoxicity and cells were incubated for 24 hours. Then, the media was removed, washed with PBS. After, removed the PBS, 200µl of trypsin-EDTA solution was added and incubated at 37°C for 3-4 minutes. Later, cells were added to 2 ml of culture media into 12 x 75 mm polystyrene tubes and centrifuged the tubes. The supernatant was decanted carefully. Cells were later washed twice with PBS and decanted the PBS completely. The contents were later incubated with 0.5 ml BD Cytofix/Cytoperm solution for 10 minutes and washed with 0.5% bovine serum albumin (BSA) in 1 X phosphate-buffered saline (PBS) and 0.1% sodium azide. Then, the cells were treated with 20µl of Anti–Caspase-3 antibody, mixed thoroughly and incubated for 30 minutes in the dark at room temperature (20° to 25°C). Then, the cells were washed with 1X PBS with 0.1% sodium azide, 0.5 mL of PBS was added and thoroughly mixed and Caspase 3 expression were analyzed by using flow cytometry [9].

Results and Discussions

Cytotoxicity of various extracts of the *I. frutescens*

Cytotoxic activities of the leaf extracts of *I. frutescens* against HeLa cell line was screened by MTT assay. In order to analyzed the anticancer activity of *I. frutescens* on HeLa cell line, cells were treated with various concentrations of 5 different leaf extracts of the *I. frutescens.* The IC_{50} was determined for each extract. Cytotoxicity of the leaf extracts (petroleum ether, ethyl acetate, chloroform, ethanol and water) against HeLa cell line showed positive results (Table 1 and Fig. 1). Among the different solvent extracts, the Chloroform leaf extract exhibited better cytotoxicity with IC_{50} value 188.6µg/ml against HeLa cell lines. Hence, chloroform leaf extract at a concentration corresponding to their IC_{50} value was used for the further studies. A comparative account on cytotoxic effect of 14 herbal plants tested on HeLa cell lines by Artun *et al.,* (2016) [4] reveled that only 4 methanolic extracts of *Cotinus coggygria Scop., Rosa damascena Miller, Colchicum sanguicolle K.M. Perss,*

and *Centaurea antiochia Boiss* showed highest cytotoxicity with IC_{50} value of 293µg/mL, 265µg/mL, 2µg/mL, 427µg/mL respectively. Thavamani *et al.*, (2013) [16] reported, the methanolic leaf extract of *Cocculus hirsutus* and *Cissampelos pareira* showed better inhibition of HeLa cells with IC_{50} value 111µg/ml and 129.3µg/ml respectively. Methanolic root extract of *I. frutescens* showed significant anticancer activity on four cancer cell lines like MCF-7 (Human breast cancer cell line), SGC-7901 (Human gastric cancer cell line), BEL-7402 (Human hepatocellular carcinoma cell line) and SPC-A-1 (Human lung cancer cell line) with IC50 values 163.5±3.58, 112.4±1.85, 156.3±2.95 and 142.6±2.60 respectively [11]. During the present investigation, potential of the chloroform leaf extract showed significant cytotoxicity with IC50 value 188.6µg/ml against Hela (Cervical cancer cell lines). Hence this chloroform leaf extract was tested for further analysis. In addition, sample treatment and standard drug (25 µM) led to morphological changes by inhibiting the of Hela cervical cancer cell lines, whereas no inhibition was observed on untreated cell lines (Fig 2). The IC_{50} value was determined by using Graphpad prism version 6.0.

Table 1: IC50 of the leaf extracts of *I. frutescens* against HeLa cell lines as determined by MTT assay. IC50- Half maximal inhibitory concentration.

Solvents	IC_{50} µg/ml
PE	298
C	188.6
EA	319.5
E	219
W	317.5

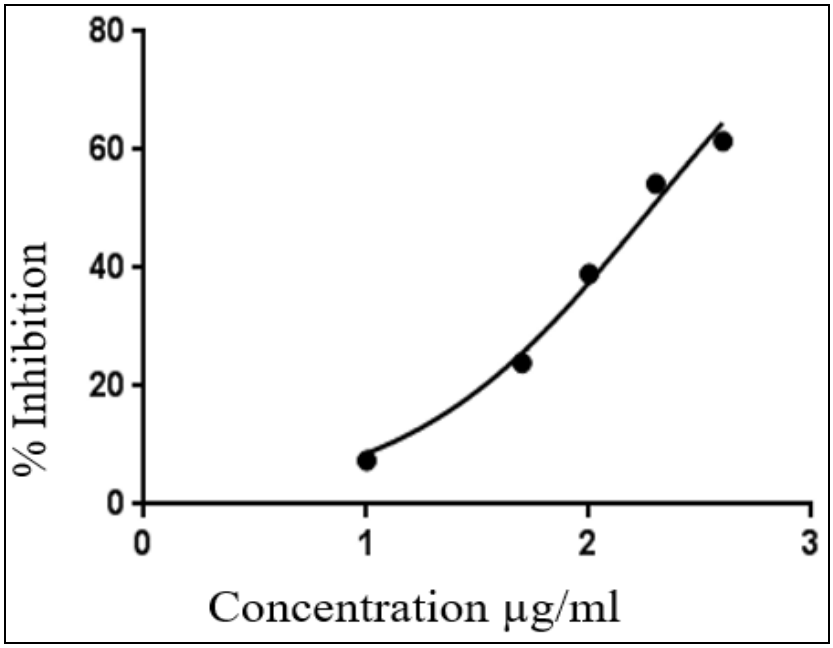

Fig 1: A graph showing percentage of inhibition at different concentration of chloroform leaf extract. Calculation and graph were prepared using Graph Pad Prism Version 6.0.

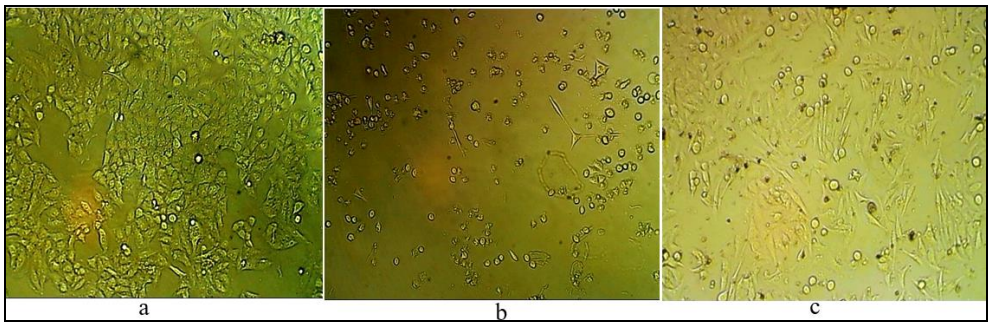

Fig 2: (a) Untreated HeLa cell, (b) HeLa cells treated with Camptothecin and (c) HeLa cells treated with Chloroform leaf extract, Fig b and c showed morphological changes of the HeLa cells after treatment with standard and chloroform leaf extract of *I. frutescencs.*

Cell cycle analysis by Pi staining

To examine the impact of chloroform leaf extract of *I. frutescens* on cell cycle of the HeLa cells, the cells were treated with chloroform leaf extract at a concentration 188.6µg/ml. At which phase of cell cycle, the cells were killed or growth arrested in HeLa cells was determined by using Propidium Iodide (PI) staining and was analyzed by Flow cytometry. The results indicated that in comparison with standard, 54.31% of HeLa cells arrest in G2/M phase by chloroform leaf extract and 65.6% of cells arrest in sub G0/G1 phase by standard leads to by DNA fragmentation reflecting apoptotic cell death (Fig. 3 and Table 2).

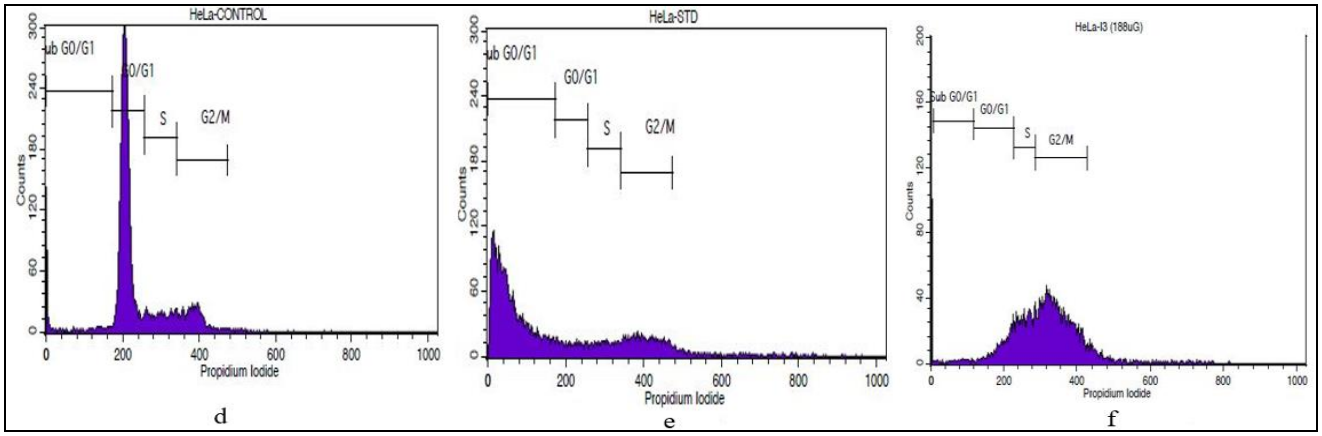

Fig 3: d: Untreated Hela cells, e: HeLa cells treated with standard (Camptothecin) and f: HeLa cells treated with Chloroform leaf extract. The cells were stained with PI staining and analyzed by flow cytometry.

Table 2: U-Untreated, CPT-camptothecin (standard) and IF *(I. frutescens)* - chloroform leaf crude extracts of *I. frutescens*, in all samples showed varying degrees of percentages of cell cycle phase distribution of HeLa cells.

Sample	Percentage of cells gated in different phases			
	Sub G0	**G0 / G1**	**S**	**G2 / M**
UT (Fig-d)	4.37	70.69	11.70	13.27
CPT (Fig-e)	65.6	7.69	8.03	16.12
IF (Fig-f)	1.11	11.60	20.59	54.31

In vitro root extract of *Menyanthes trifoliata* was able to inhibit cell growth, arresting cell cycle in the G2/M phase, inducing the apoptosis in human glioma cells (Brain tumor) was reported by Kowalczyk *et al*., (2019) [10]**.** Zeng *et.al.*, (2018) [18] investigated the cytotoxic activity of caffeic acid n-butyl ester (CAE) against the A431 skin cancer cell line determined by MTT assay and also reported CAE was associated with apoptosis and cell cycle arrest of A431 cells in G2 phase.

***I. frutescens* induces the apoptosis against HeLa cell lines**

Chloroform leaf extract of this plant was subjected to induction of apoptosis on HeLa cell line by using Annexin V and PI double stain. Based on the MTT assay, HeLa cells were treated with chloroform leaf extract at a concentration 188.6µg/ml. The percentage of cells analyzed immediately after addition of PI which can be classified in to four categories. The populations of cells residing in the Annexin V+/PI- and the Annexin V+/PI+ quadrants were determined as early (Low Right) and late apoptotic cells **(**Upper Right), respectively. The Annexin V-/PI- and the Annexin V-/PI+ quadrants of the density plot were represented as live cells (Low Left) and necrotic cells **(**Upper left) respectively (Wlodkowic *et al.*, 2009) [17]. In results indicated that, comparison between the untreated and standard, chloroform leaf extracts were showed varying degrees of percentages of apoptotic cells (early apoptotic and late apoptotic). Chloroform leaf extracts of *I. frutescens* when treated with the Hela cells showed increased in percentage of early apoptotic cells (Low Right) 55.74% and late apoptotic cells 31.51% **(**upper Right). In standard, showed the percentage of early apoptotic cells (Low Right) to 46.24% and late apoptotic cells 32.02% **(**upper Right) as shown in (Fig. 4 and Table 3). These results clearly revealed that the chloroform leaf extracts of *I. frutescens* induces the Apoptosis against HeLa cell lines. Zeng *et al*., (2018) [18] reported the percentage of apoptotic cells increased from 1.25% in the control to 46.37% in the 40µM CAE-treated (caffeic acid n-butyl ester) group against the A431 skin cancer cell line.

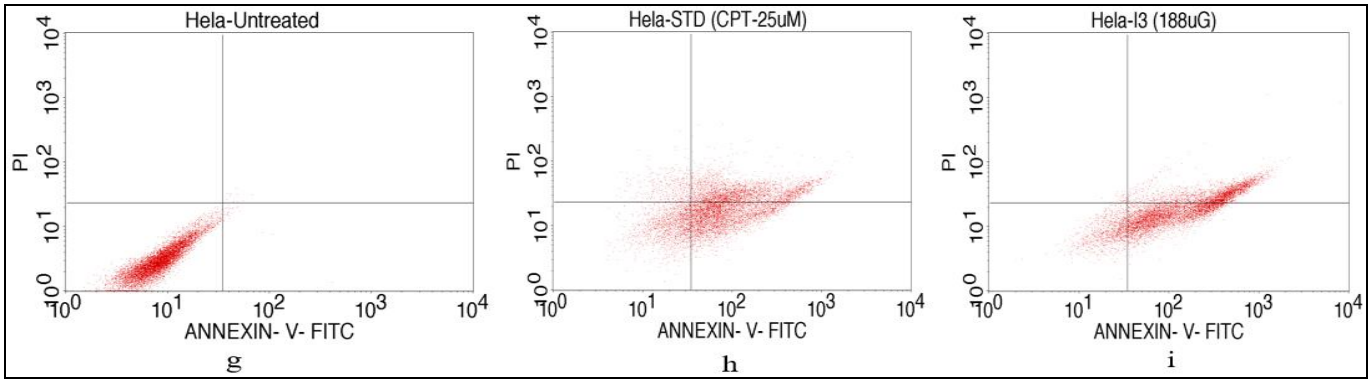

Fig 4: (g) untreated, (h) standard and (i) Apoptosis induction effect of chloroform leaf extract at IC50 (188.6µg/ml) on HeLa cells for 48 hours determined by Annexin V-FITC/PI flow cytometry. Dot plot diagrams show percentage of cell populations divided to viable (lower left quadrant: Annexin V and PI negative), early apoptotic (lower right quadrant: Annexin V positive and PI negative), late apoptotic (Upper right quadrant: Annexin V and PI positive), and necrotic cells (upper left: Annexin V negative and PI positive).

Table 3: UT-untreated, CPT-camptothecin (standard) and IF *(I. frutescens)*-chloroform leaf extracts of *I. frutescens,* in all samples showed varying degrees of percentages of apoptotic cells (the percentage of the necrosis, early apoptotic and late apoptotic cells).

Samples	Percentage of cells gated in different quadrants			
	Live cells	Early apoptosis	Late apoptosis	Necrosis
UT (Fig-g)	99.60	0.29	0.11	0.00
CPT (Fig-h)	17.69	46.24	32.02	4.05
IF (Fig-i)	12.36	55.74	31.51	0.39

Caspase 3 expression studies by flow cytometry

The aim of this study was to investigate whether the chloroform leaf extract of *I. frutescens* has any influence on caspase 3 expression leading to apoptosis in HeLa cells or not. The percentage of cells expressed caspase 3 analyzed by flow cytometry. In the present study untreated cells, cells treated with standard (Camptothecin) and chloroform leaf extract were compared and it found to exhibit the percentage of cells express moderate caspase 3 i. e., 29.23% and 31.35% in standard and chloroform leaf extract treated cells in which HeLa cancer cells become apoptotic (Fig: 5 and Table 4). These results indicated that the majority of cells followed caspase- independent pathway of apoptosis. Caspases are a family of aspartate-specific cysteine proteases (catalytic enzyme) present in the cell cytoplasm in an inactive form (known as procaspases) classified in to initiators (caspase-2, 8, 9, 10), inflammatory caspases (caspase-1, 4, 5) and effectors or executioners (caspase-3, 6, 7). Caspase-3 were the most important of the executioner caspases and common to both the extrinsic and intrinsic death pathways. This is activated by any of the initiator caspases and in endonuclease CAD, Caspase-3 specifically activates [8]. Parsaee, *et al*., (2013) [14] reported that the methanolic root extract of *Salvia chorassanica* was induced apoptosis by increasing the activity of caspases 3 and caspases 8 against HeLa Cell line through the extrinsic pathway.

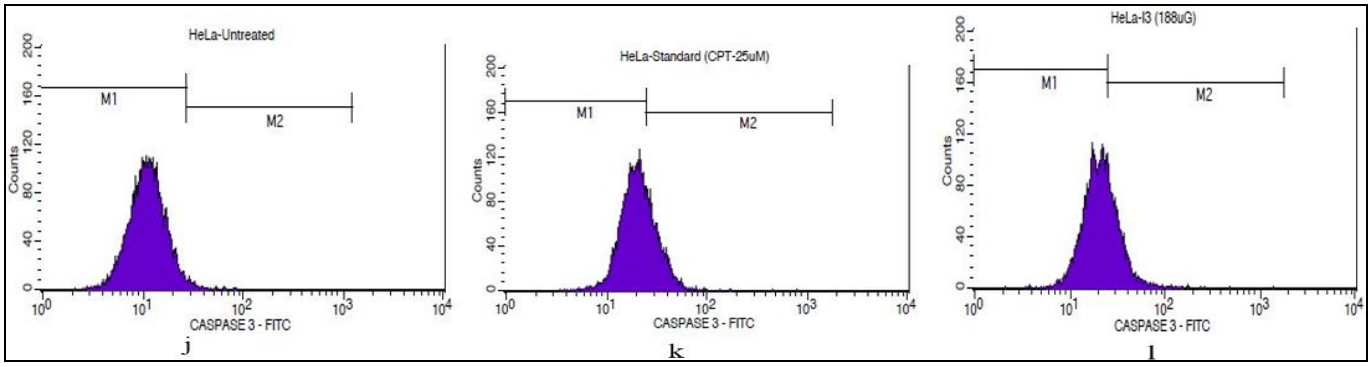

Fig 5: Caspase 3 Expression Studies analyzed by flow cytometry using Caspase 3 biomarker. Untreated Hela cells (j), HeLa cells treated with standard (Camptothecin) (k) and Chloroform leaf extract (l) showed varying degree of Caspase 3 Expression.

Table 4: UT- untreated, CPT-camptothecin (standard) and IF *(I. frutescens)* - chloroform leaf crude extracts of *I. frutescens,* in all samples showed varying degrees of percentages of the expression of caspase 3.

Samples	Percentage of cells express caspase - 3	
	M1 (devoid of caspase-3)	M2 (cells with caspase-3)
UT (Fig-j)	98.79	1.25
CPT(Fig-k)	71.69	29.23
IF (Fig-l)	69.63	31.35

Conclusions

During the present investigation, the cytotoxicity of *I. frutescens* leaf extracts (petroleum ether, ethyl acetate, chloroform, ethanol and water) were tested by MTT assay against HeLa cell line. Chloroform leaf extract showed significant cytotoxicity with IC_{50} value of 188.6µg/ml compared to other extracts. It has been demonstrated that dose-dependent inhibition on the growth of the HeLa cells by apoptosis is due to caspase 3 expression. The present study also recorded the impact of *I. frutescens* leaf extract on the cell cycle phase of HeLa cells in G2 phase leading to the arrest of more number of cells. The results of the present study demonstrated the potent cytotoxic activity of the chloroform leaf extract of *I. frutescens* against HeLa cell line. Hence, this plant can be recommended for the formulation of new therapeutic drugs of pharmaceutical industries to cu\re human cervical cancer.

References

1. Adhikari BS, Babu MM, Saklani PL, Rawat GS. Medicinal plants diversity and their conservation status in Wildlife Institute of India (WII) campus, Dehradun. Ethnobotanical leaflets,2010:1:6.
2. Anbarashan M, Padmavathy A. Ethno-medicinal plants in five sacred groves in Cuddalore district, Tamilnadu, India. Ethnobotanical leaflets,2010:14:774-780.
3. Arbyn M, Weiderpass E, Bruni L, de Sanjosé S, Saraiya M, Ferlay J *et al*. Estimates of incidence and mortality of cervical cancer in 2018: a worldwide analysis. The Lancet Global Health,2020:8(2):191-203.
4. Artun T, Karagoz A, Ozcan G, Melikoglu G, Anil S, Kultur S *et al*. *In vitro* anticancer and cytotoxic activities of some plant extracts on HeLa and Vero cell lines. Journal of BU ON.: official journal of the Balkan Union of Oncology,2016:21(3):720-725.
5. Badmus JA, Ekpo OE, Hussein AA, Meyer M, Hiss DC. Antiproliferative and apoptosis induction potential of the methanolic leaf extract of *Holarrhena floribunda* (G. Don). Evidence-Based Complementary and Alternative Medicine, 2015, 11-12.
6. Bhandary MJ, Chandrashekar KR, Kaveriappa KM. Medical ethnobotany of the siddis of Uttara Kannada district, Karnataka, India. Journal of ethnopharmacology,1995:47(3):149-158.
7. Chothiphirat A, Nittayaboon K, Kanokwiroon K, Srisawat T, Navakanitworakul R. Anticancer potential of fruit extracts from Vatica diospyroides symington type SS and their effect on program cell death of cervical cancer cell lines. The Scientific World Journal, 2019, 1-9.

8. Elmore S. Apoptosis: a review of programmed cell death. Toxicologic pathology,2007:35(4):495-516.
9. Huang Y, Ohno O, Suenaga K, Miyamoto K. Apoptosis-inducing activity and antiproliferative effect of Paeoniflorigenone from moutan cortex. Bioscience, biotechnology, and biochemistry,2017:81(6):1106-1113.
10. Kowalczyk T, Sitarek P, Skała E, Toma M, Wielanek M, Pytel D *et al*. Induction of apoptosis by *in vitro* and *in vivo* plant extracts derived from *Menyanthes trifoliata* L. in human cancer cells. Cytotechnology,2019:71(1):165-180.
11. Kumar Singh N, Pratap Singh V. Anticancer activity of the roots of *Ichnocarpus frutescens* R. Br. and isolated triterpenes. Pakistan journal of pharmaceutical sciences,2014:27(1):187-191.
12. Kumarappan C, Srinivasan R, Jeevathayaparan S, Rajinikanth R, Naveen Kumar HS, Senthilrajan S *et al*. *Ichnocarpus frutescens*: A valuable medicinal plant. Pharmacol Online,2015:19:18-37.
13. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. Journal of immunological methods,1983:65(1-2):55-63.
14. Parsaee H, Asili J, Mousavi SH, Soofi H, Emami SA, Tayarani-Najaran Z. Apoptosis induction of Salvia chorassanica root extract on human cervical cancer cell line. Iranian journal of pharmaceutical research: IJPR,2013:12(1):75.
15. Thangarajan S, Perumal S, Chinthamony AR, Paramasivam R, Balasubramanian V, Martin S *et al*. Chemomodulatory effects of *Ichnocarpus frutescens* R. Br against 4-vinylcyclohexane induced ovarian cancer in swiss albino mice. Journal of Acute Disease,2013:2(2):151-155.
16. Thavamani BS, Mathew M, Dhanabal SP. *In vitro* cytotoxic activity of menispermaceae plants against HeLa cell line. Ancient science of life,2013:33(2):81.
17. Wlodkowic D, Skommer J, Darzynkiewicz Z. Flow cytometry-based apoptosis detection. Humana Press, Totowa, NJ, 2009, 19-32.
18. Zeng N, Hongbo T, Xu Y, Wu M, Wu Y. Anticancer activity of caffeic acid n-butyl ester against A431 skin carcinoma cell line occurs via induction of apoptosis and inhibition of the mTOR/PI3K/AKT signaling pathway. Molecular medicine reports,2018:17(4):5652-5657.