Research Article (Open access)

Anticancer and Cytotoxic Potential of Turmeric (*Curcuma longa*), Neem (*Azadirachta indica*), Tulasi (*Ocimum sanctum*) and Ginger *(Zingiber officinale*) Extracts on HeLa Cell line

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ABSTRACT- The Human papillomavirus (HPV) is the single etiological factor in cervical cancer, contributing to neo-plastic progression through the action of viral oncoproteins, mainly E6 and E7. Cervical cancer remains the second most common cancer in women worldwide with India as a major contributor to the global burden with an annual incidence of 132,000 new cases and mortality rate of 74,000 deaths annually. In this study turmeric, neem, tulasi and ginger were selected as natural anticancer drugs. The objective of the study was to analyze the anticancer property of turmeric (*Curcuma longa*), neem (*Azadirachta indica*), tulasi (*Occimum sanctum*) and ginger (*Zingiber officinale*) on HeLa cells. Turmeric, neem, tulasi and ginger capsules (Himalaya's Company) were used and aqueous and methanolic extracts of the turmeric, neem, tulasi and ginger were obtained using a soxhlet extraction. To check the efficacy of these drug MTT assay was performed that determined (%) viability and/or cytotoxicity. IC_{50} of aqueous turmeric, neem, tulasi and ginger extracts in case of HeLa cells were 17.8, 22, 79.4, 27.86, respectively and in case of methanolic turmeric, neem, tulasi and ginger extracts 17, 7.35, 75.24 and 16.1, respectively. To confirm apoptosis as the sole reason behind cell death immunofluorescence based apoptosis assay was performed using TALI image based cytometer. The study has led to postulate hypothesis that natural drugs e.g. turmeric, neem, tulasi and ginger are potent anti-cancer compound that are capable of inhibiting the growth of immortal cells by apoptosis.

Key-Words: Cervical cancer, Human papillomavirus (HPV), Oncoproteins E6 and E7, Natural compounds, HeLa cell line (adherent), Cell viability and MTT assay, Apoptosis assay

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INTRODUCTION

Human papillomavirus (HPV) infection is found in more than 95% of cervical cancer. In India, cervical cancer is a leading cancer among women with annual incidence of about 130,000 cases and 70-75,000 deaths [1]. Expression of HPV E6 and E7 oncogenes are one of the high risk factor for the initiation and maintenance of cervical cancer. Its deregulated expression leads to disruption of normal cell cycle regulation, abrogation of apoptosis and genetic instability.

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Furthermore, repression of E6 and/or E7 may induce cancer cells to undergo apoptosis or senescence [2,3]. In recent years, natural dietary agents have drawn a great deal of attention because of their potential ability to suppress cancers as well as reduce the risk of cancer development. There are more than one thousand species that have been found to possess significant anticancer properties [4,5]. The tremendous ability of natural products to act as effective scaffolds and bind bewildering types of protein domains and folding motifs makes them effective modulators of various cellular processes, contributing to immune responses, signal transduction, cell division and apoptosis [6,7]. The cytotoxic activity of natural compounds to cervical cancer cells in a concentration and time dependent manner were selectively more in HPV16 and HPV18 infected cells compared to non-HPV infected cells [11]. Natural compounds analogs have effective binding with different active sites on HPV16 E6 protein, ideal target for restoring

the tumor suppressor function of p53 and thus allowing the apoptosis of infected cells [8,9].Therefore, proposed study aim to identify potential natural compounds and their derivatives targeting HPV16 & 18 on cervical cancer cell line (HeLa) using the MTT cell proliferation assay. [10,12]

MATERIALS AND METHODS

Culturing Cell line and Media Conditions

The human cervical cancer cell lines (HeLa) were obtained from National Centre for Cell Sciences, Pune (NCCS), India. The cells were maintained in DMEM growth media with HEPES, 10% FBS, Non-essential amino acids, Sodium bi-carbonate and antibiotic cocktail (Penicillin, Streptomycin, Amphotericin-B (HIMEDIA Laboratories Pvt. Ltd.) in a humidified atmosphere of CO_2 incubator at 37ºC and 5% CO_2 with 95% air. Cultured cells were replaced with fresh media after 2-3 days. When cells were reaching up to 90% confluences considered for proliferation assay. Cell viability was assessed by trypan blue dye exclusion assay. Percent viability was calculated by following formula:

Cell viability assay= %viability= (Viable cells / Total cells) x 100

Extraction of Phytochemicals

Himalaya's company capsules were used in selected natural compound's material. About 10gm of the powdered sample of each compound were weighed into 150 ml of aqueous extract (AE) and methanol extract (ME) in a soxhlet apparatus separately. Vaporization of methanol and water were allowed until the extract appeared from color to colorless and the above process was repeated for several times, until the sufficient amount of extract is produced. Extracted samples were kept in to hot air oven overnight at 60°C and100°C, so that the methanol and water gets evaporated. The concentrated extract of each plant was stored at 4°C until when required for use.Yield of extraction was calculated using the following formula:

$$\% \text{ Yield of extraction} = \frac{\text{Weight of final dried extract}}{\text{Weight of initial powder taken for extraction}} \times 100$$

Phytochemical analysis was carried out in natural compounds to determine the presence or/and absence of alkaloids, flavonoids, glycosides, phenol, saponin and tannin.

Table 1: Method and indication for phytochemicals

Test	Method	Indication
Alkaloid	1gm of powder + 1.5% HCl + Few drops of Wagner"s Reagent	Brown precipitate indicated presence of Alkaloid
Flavanoid	2 gm of powder + 5ml of dilute ammonia solution + few ml of concentrated Sulfuric acid solution	Yellow color indicated presence of flavanoid
Glycoside	1 gm of powder + 1ml of Distilled Water + few ml of 1N Sodium Hydroxide solution	Yellow color indicated presence of Glycoside
Phenol	2 gm of powder + 2ml Distilled water + Few drops of 10% Ferric Chloride solution	Blue/Green Color indicated presence of phenol
Saponin	1 gm of powder + 5ml Distilled Water	Honey comb like froth indicated presence of saponin
Tannin	Few gm of powder + few drops of 5% Ferric Chloride solution	Bluish black color indicated presence of tannin

Preparation of AE and ME Sample for MTT Assay

ME and AE were dissolved in methanol and PBS solution, respectively to get a concentration of 1 mg/10 µl. From this, dilutions were prepared to obtain final drug concentration ranging from100 µg to100 ng.

MTT Assay Procedure

This colorimetric assay is based on the capacity of mitochondria succinate dehydrogenase enzymes in living cells to reduce the yellow water soluble substrate 3 (4,5-dimethylthiazolyl)-2,5-diphenyl-tetrazolium bromide (MTT) into an insoluble purple colored formazon product which is measured spectrophotometrically. This formazon production is directly proportional to the viable cell number and inversely proportional to the degree of cytotoxicity. HeLa cells were seeded in 96-well plates at a density of 1×10^4 cells/well in DMEM. After 24 hours of seeding at 37°C and 5% CO_2 condition. Stock solutions of 1mg/10µl were prepared from each and every extracted sample. Samples were further diluted in a range of 100 µg to 100 ng. Aqueous and methanolic extracts were added in increasing concentration in the wells. After incubation, 20 µl of MTT reagent was added into each well. Incubated for 4 hrs followed by monitoring using inverted microscope. Solubilizing reagent was added to solubilize the formazon crystals (Hi-Media kit based procedure). The resulting MTT products were determined by measuring the absorbance at 590 nm using ELISA reader. IC50 values were calculated as the concentrations that show 50% inhibition of HeLa cells proliferation and it was calculated using following equation:

$y = x^2+x+1$ where, y = % viability and x = log [conc.]

Apoptosis Assay Procedure

Exposed cells to drug were assessed for apoptosis and cells not exposed to test drug were considered as control.1X Annexin binding buffer was prepared by diluting the Component C in deionized water. Cells were re-suspended in 1x Annexin binding buffer, so that there was at least 100 µL of cells per individual assay at a concentration of approximately 5×10^5 to 5×10^6 cells/ml. To each 100µL of sample, 5 µL of component A was added and mixed well. The cell-annexin V mixture was incubated at room temperature in the dark for 20 minutes. The cells were centrifuged and re-suspended in 100 µL of ABB.1 µL of Tali® component was added to each 100 µL sample and mixed well. Samples were incubated at room temperature in the dark for 1–5 minutes. 25 µL of the stained cells were used for counting and further analysis (Invitrogen kit based).

RESULTS

Turmeric, neem, tulasi, ginger was shown that they contain Alkaloid, Flavonoid, Phenolic, Tannin, Saponin and Glycoside.

Table 2: Phytochemical analysis of Turmeric, Neem, tulasi, ginger

Phytochemicals	Turmeric	Neem	Tulasi	Ginger
Alkaloid	+	+	+	+
Flavonoid	+	+	+	+
Tannins	-	+	+	-
Saponins	-	+	+	-
Phenolic	+	+	+	+
Glycoside	-	+	+	+

Table 3: Dry weight (yields) of Aqueous and Methanolic Turmeric, Neem, Tulasi and Ginger extract

S. No.	Extracted sample	Final wt. of the aqueous extracted sample (gms)	Final wt. of the methanolic extracted sample (gms)
1	Turmeric	0.15	0.46
2	Neem	1.37	0.69
3	Tulasi	0.45	0.97
4	Ginger	0.43	0.52

Table 4: IC50 value of Turmeric, Neem, Tulasi and Ginger in case of HeLa cells

Compound	Extraction	IC50 value of HeLa cells
Curcumin	Methanolic	17
	Aqueous	17.8
Ginger	Methanolic	16.01
	Aqueous	27.86
Neem	Methanolic	7.35
	Aqueous	22
Tulasi	Methanolic	75.24
	Aqueous	79.4

IC50 value of aqueous and methanolic turmeric, neem, tulasi and ginger extract on HeLa cells were as above. Different extracted drug concentrations were used and found decreasing cell viability with increasing drug concentration. Also curcumin, neem and tulasi and ginger aqueous extract show higher cytotoxicity value and among four compounds neem gave lower cytotoxicity value and higher viable cell counts.

Table 5: Cytotoxicity on HeLa cells by Aqueous extract of Turmeric

Conc. (µg)	% Viability	% Cytotoxicity
Cell control	100	0
0.1	86	14
1	79	21
5	66	34
10	54	46
25	46	54
50	35	65
100	25	75

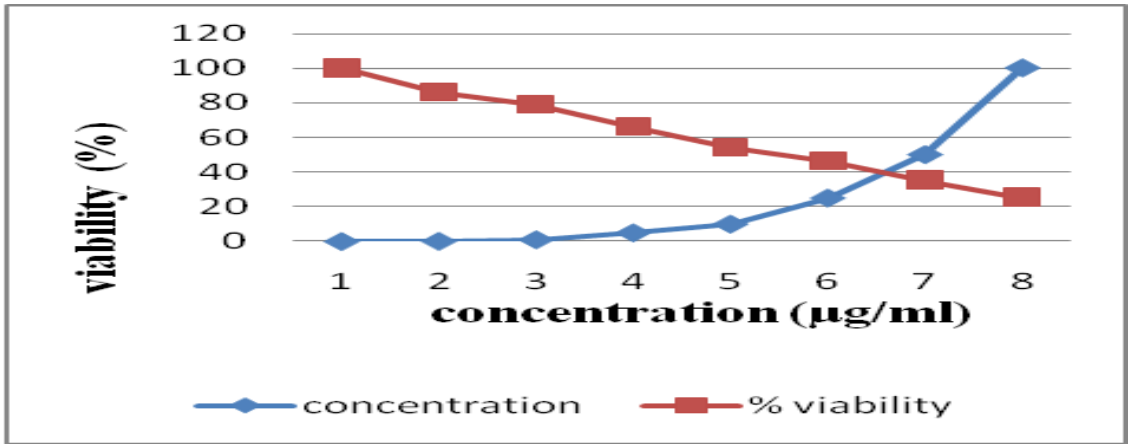

Fig. 1: Cytotoxicity on HeLa cells by aqueous extract of turmeric

The cytotoxicity of the aqueous turmeric extract in HeLa cells was maximum at a drug concentration of 100 µg/ml i.e. 75% and lowest viability value found was 25% at 100 µg/ml of drug concentration. Also obtained IC50 value was 17.8 µg /ml.

Apoptosis Assay Indication

Apoptosis assay for selected natural compounds in HeLa cells were as follows. Green fluorescence determined (%) apoptosis.

For Turmeric: $1.44x10^7$ cells/ml out of $1.45x10^7$ cells/ml show green fluorescence (apoptosis) and 1.31×10^5 cells/ml show no green fluorescence (no apoptosis). So the resulting output data indicated that 99% of cell deaths were due to apoptosis.

For Neem: 2.68×10^6 cells/ml out of $2.68x10^6$ cells/ml show green fluorescence (apoptosis). So the resulting output data indicated that 100% of cell deaths were due to apoptosis.

For Tulasi: 2.92×10^6 cells/ml out of $2.92x10^6$ cells/ml show green fluorescence (apoptosis). So the resulting output data indicated that 100% of cell deaths were due to apoptosis.

For Ginger: $>1.5x10^7$ cells/ml out of $>1.5x10^7$ cells/ml show green fluorescence (apoptosis). So the resulting output data indicated that 100% of cell deaths were due to apoptosis.

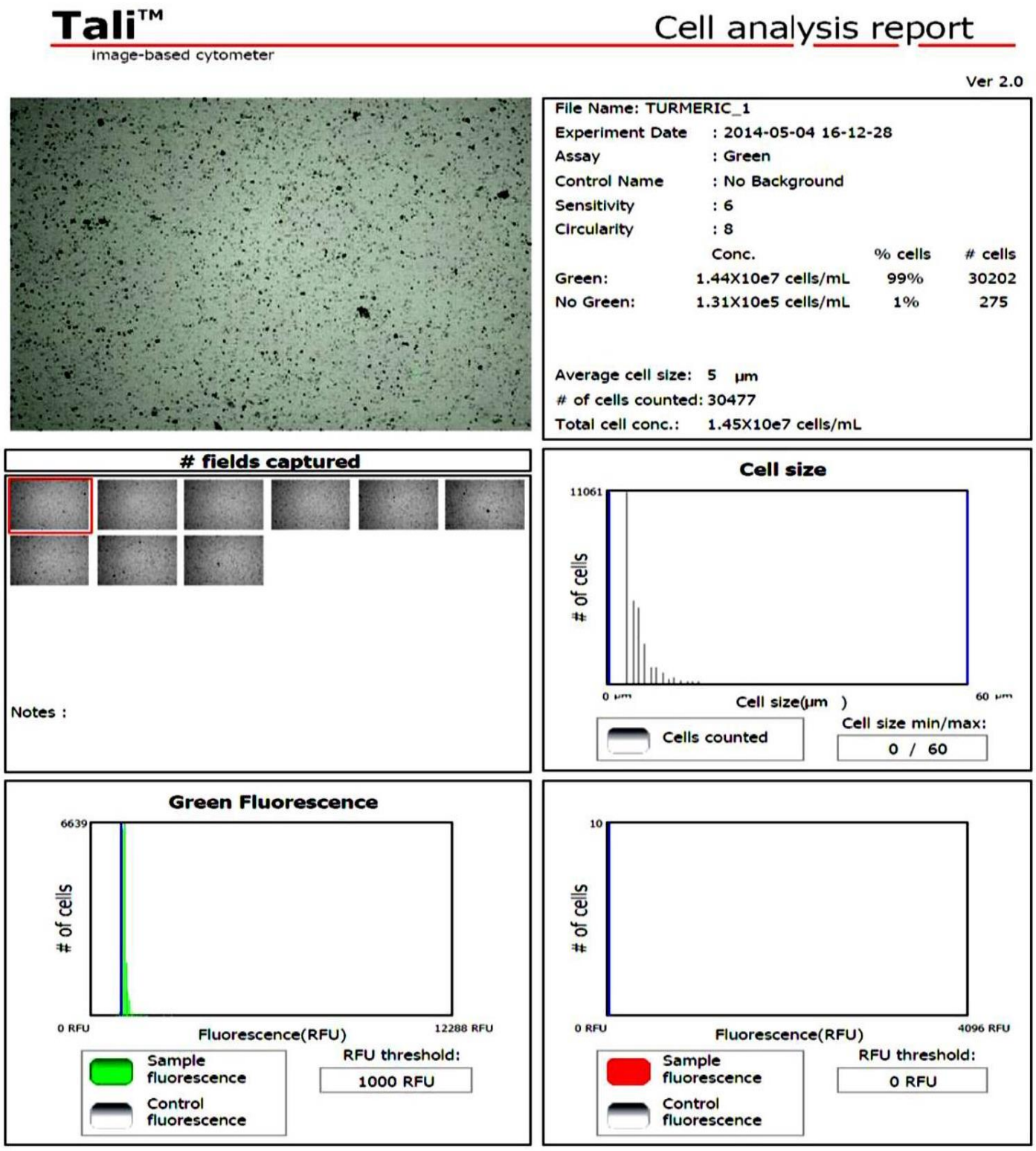

Fig. 2: Tali image based flow cytometer revealed 99% cell death due to apoptosis in case of turmeric

Immuno fluorescence Study for Validation/Confirmation

Apoptosis assay was carried out by Annexin-V and PI staining Cells exposed to 45mM concentration were stained using FITC labeled anti-Annexin V Ab and propidium iodide to stain nuclei. The images were captured using specific filters and in the phase contrast mode for total cellularity estimation. Then the capture images were superimposed to confirm the apoptosis and necrotic cell death. Cells stained in green only shown apoptotic cell death, whereas cells stained with only red nuclei suggestive of necrotic cells. Cells stained with both green and red shown early apoptotic cells. However, unstained cells are live and with intact membrane.

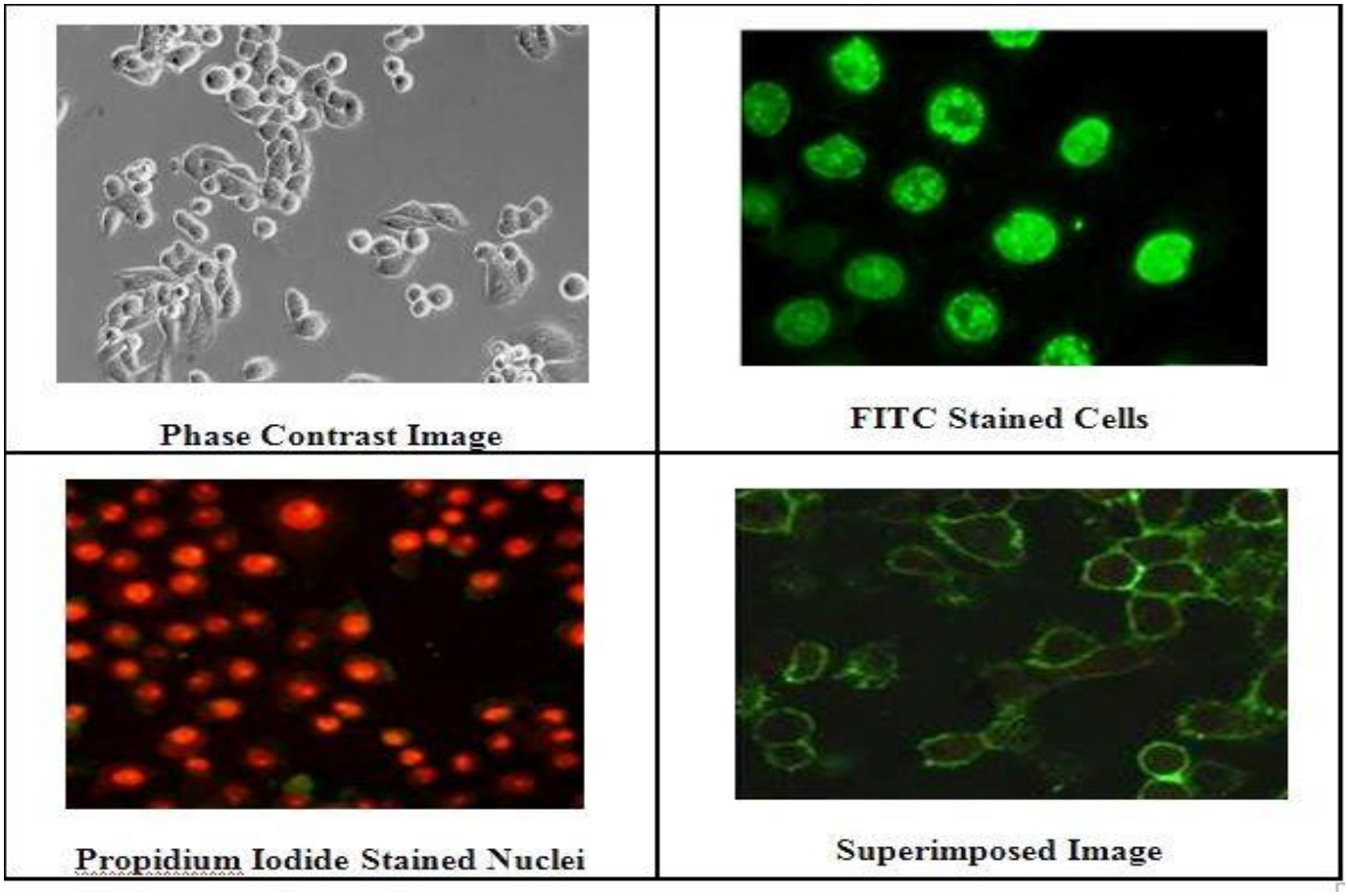

Fig. 3: Apoptic assay showed Annexin-v and PI stained cells of HeLa

DISCUSSION

HPV infection is closely associated with the development of more than 95% of cervical cancer. Natural compounds are gaining interest as potential cancer therapeutics including for the treatment of cervical cancer. The present need is to develop drugs that can be potentially target cancer cells by means of their inherent difference to normal cells [13]. The present study demonstrated the promising cytotoxic and anticancer activities of the aqueous and methanolic extract of turmeric, neem, tulasi and ginger against HeLa cell lines. Different extracts of the plant exhibited different activity on different cell lines. This selectivity could be due to the sensitivity of the cell line to the active compounds in the extract or to tissue specific response. Phytochemicals present in plants protect the cells from oxidative damage and are responsible for the death of cells [14,15]. In these study, results of phytochemical analysis shown turmeric, neem, tulasi and ginger contain alkaloids, flavonoids, and glycosides, saponin, tannin and phenolics [16]. Different *in vitro* cytotoxicity assays with different endpoints have been employed for screening of potential natural compounds extract preparation for their anticancer activities. The most commonly used assays involve the use of dye stains that include MTT. This dye has some indicator properties allowing them to reveal ongoing cellular processes, providing indirect measure of mitochondria function [17-19].

In this study aqueous and methanolic extracts of turmeric, neem, tulasi and ginger were screened for its cytotoxicity at different concentrations to determine the IC50 value. A chart was plotted using the % cell viability in Y-axis and concentration of natural compounds extracts in X-axis. IC50 of aqueous turmeric, neem, tulasi and ginger extracts for HeLa cells were 17.8,22,79.4,27.86, respectively and in the case of methanolic turmeric, neem, tulasi and ginger extracts 17,7.35,75.24 and 16.1, respectively. Also comparing our results with others data it was clearly indicated that curcumin and other natural products can be cytotoxic to cervical cancer cells in a concentration-dependent and time-dependent manner and It also induced apoptosis in cervical cancer cells [20,21]. The cytotoxic activity was selectively more in HPV16 and HPV18 infected cells compared to non-HPV infected cells [22,23]. Apoptosis assay was performed to confirm the cause of death of the cancer cells. In this study apoptosis assay result obtained 99% death of cells was due to apoptosis and remaining 1% of death might have been due to necrosis, or any other cell inhibiting factor. In the present study, 99% cell death in case of turmeric and 100% cell death obtained in case of neem, tulasi and ginger. The study has led to postulate hypothesis that turmeric, neem, tulasi and ginger are potent anti-cancer compound that are capable of inhibiting the growth of immortal HeLa cells by apoptosis [24,25].

CONCLUSIONS

In our study, we deeply believe that the cross killing occurred due to cytotoxic activity against the Cervical cancer- HeLa cell line. The results of the present study demonstrated the potent cytotoxic activity of the aqueous and methanolic extracts of turmeric, neem, tulasi and ginger and phytochemical constituents are the major com ponents which are responsible for the potential cytotoxic activity. Even though there was increase in the cell growth inhibition or cell viability decreased when the concentration of drug extract was increased. To confirm apoptosis as the sole reason behind cell death immune fluorescence based apoptosis assay was performed using TALI image based cytometer. Further research is also needed for proving with other cancer models and human beings with also isolating the active principle of natural compounds. The anticancer property of selected natural compounds will provide useful information on the possible application in the prevention and treatment of cervical cancer.

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