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Research Article

In-Vitro Cytotoxic and Apoptotic Effect of *Wrightia tinctoria* Roxb. Alcoholic Extract/Fractions

Singh Charanjeet[1*], Ahuja Dharmendra[2], Mehta SC[2]

[1]*Faculty of Pharmaceutical Science, Jayoti Vidyapeeth Women's University, Jaipur, India*
[2]*Faculty of Pharmaceutical Science, Jayoti Vidyapeeth Women's University, Jaipur, India*

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ABSTRACT

The ayurvedic medicine system of prescription suggests *Wrightia tinctoria* Roxb. (Apocynaceae), for the treatment of tumors. The investigation is required as malignant growth is the second most basic reason for death on the planet; the greater part of the customary medications have appeared, minimal effort approaches, and higher harmfulness of the current drugs. The present examination means to decide the method of cell death instigated by the alcoholic extract fractions of *Wrightia tinctoria* on human malignant growth cell lines. Amongst crude alcoholic extract of *Wrightia tinctoria* Roxb. investigate for potential anticancer action, there are two fractions selected for investigation these are WTD (dichloromethane fraction) and WTE (ethyl acetate fraction) hey were found to have capable cytotoxic potential on all cancerous cell lines contemplated by MTT test and SRB test. Ethyl acetate fraction found to have increasingly powerful action in A549, U343HeLa, BT549 and HCT15 cells lines. Strangely, ethyl acetate was less cytotoxic in HBL-100 cell line, demonstrate the particular action towards malignant growth cells. AO/EB staining assay and Hoechst-33342 staining assay demonstrate membrane blebbing, consolidated and fragmented nuclei upon treatment with ethyl acetate and dichloromethane fraction in A549, U343 and HeLa cell lines. DNA fragmentation stepping stool and genomic DNA discontinuity were seen with DNA fragmentation test dependent on gel electrophoresis and COMET test by fluorescent microscopy method. Essentially, cell cycle examination by flow cytometer demonstrates mutilation of ordinary cell cycle and expanded subG_0phase. Ethyl acetate fraction was found genotoxic by micronuclei development. These outcomes show that both fractions instigate apoptosis however not cells decomposition in the malignant growth cells. This plant keeps potent anticancer activity in vivo and further researches are to recognize the responsible phytoconstituents and mechanism of action.

Keywords: - MTT, SRB, *Wrightia tinctoria* Roxb, Cytotoxicity, Apoptosis.

INTRODUCTION

Apoptosis is cellular suicide or automated cell death by activation of the cellular pathway maintained inside the cell. Recently, the relationship between apoptosis and cancer has confirmed that the process of transformation, progression and metastasis of the tumor involves changes modifications of typical apoptotic pathway[1]. Apoptotic also provides some evidence on the efficacy of cancer therapy and numerous chemotherapeutic agents reported in the case of its anti-tumor effect including tumor cell apoptosis. Therapeutic plants have been utilized as solutions for human illnesses for quite a long time. The explanation behind utilizing them as drug lies in the way that they contain compound segments of the healing worth[2]. The value of healing plants in some chemicals (usually secondary metabolic products) is a specific physiological work in the human body.

Wrightia tinctoria (Roxb.) having a place with the family Apocyanaceae is normally known as Dudhi. It is an important restorative plant utilized broadly in the conventional frameworks of medication. Customarily, the plant has been utilized for looseness of the bowels, skin disease, fart, bilious conditions[3], jaundice, swelling conditions[4], wound curative[5], and in cancer treatment[6]. The plant is likewise utilized in the treatment of psoriasis, fever, looseness of the bowels, bleeding, and as a cure for snake poison[7]. Phytoconstituents available in the plant incorporate glycosides, steroids, triterpenoids, saponins, tannins, phenolics compounds and flavonoids[8]. An ongoing report likewise affirmed the anticancer action of whole plant alcoholic extract/fractions in different cell lines[6]. However, as far as we could possibly know, the plant fractions have not been assessed for their cytotoxicity toward malignant cell lines. We also assessed in this investigation for in vivo antioxidant activity.

Chemical prevention helps stop or reverse the malignant development of the external antioxidant supply to reestablish the oxidative balance in the primitive cells. The compounds obtained from the plants can act as good chemical protection agents by reducing oxidative stress and reducing oxidative stress that prevent the development of cancer. Furthermore, it is known that natural compounds

**Author for Correspondence: charanjeet2004@gmail.com*

act simultaneously on multiple malignancy targets. Current therapeutic chemicals have limited selectivity in malignancy cells compared to normal cells only by rapid division of cancer cells. As a result, even fast-dividing normal cells such as hematopoietic cells, hair cells and digestive cells are influenced prompting undesirable unfriendly impacts related with chemotherapy[9,10]. The lack of multiple selective and modified targets at the same time are important challenges other than the challenges in cancer treatment. The synergistic impacts of many selective plant components, safety, adequacy and efficacy are provided by changing the keys that cause intermediaries at the same time and certainly worth exploring[11,12,13]. We investigate the cytotoxic action from ethanolic extract / fractions from the entire plant of *Wrightia* in human carcinoma cell lines, non-cancerous fibroblasts and indicating possible ways of making cell death. Furthermore, the free radicals scavenging and in vivo antioxidant capacity to improve its use as a chemical protection agent is examined.

MATERIALS AND METHODS

Collection of plant material

The fresh plant was collected from Jaipur, Rajasthan in September month and authenticated by University of Rajasthan, Jaipur, Rajasthan. An authenticated specimen was submitted in the herbarium, Department of botany, University of Rajasthan, Jaipur, Rajasthan, India.

Preparation of extract

The fresh plant was harvested, rinsed under tap water and oven dried. The coarsely powder material was extracted with alcohol by using Soxhlet apparatus and solvent was removed by distillation and concentrated using a rotavapor.

Fractionation of crude extract

Crude alcoholic extract of *Wrightia tinctoria* (Roxb.) was suspended in water and after that fractionated with organic solvents by increasing polarity to get petroleum ether, dichloromethane, n-butanol, ethyl acetate and alcohol-water (i.e. hydro-alcoholic mixture)[14].

Preparation of various concentrations

Diverse concentrations of crude extract and fractions were prepared by dissolving the extract in DMSO and then adulterating it with DMEM medium under sterile conditions.

Cell culture conditions

Cancer cells were maintained in Dulbecco's modified eagle medium (DMEM) with1000 mg/mL of glucose, supplemented with 10% FBSFBS (fetal bovine serum) and penicillin/streptomycin-L-glutamine and cultured in a humified atmosphere of 5% CO_2 and 95% air at thirty-seven degree Celsius in incubator[15].

Screening of cytotoxicity/anticancer action

Malignant growth cell line was utilized for the assurance of cytotoxic action. The cells are seeded at nineteen-six wells with the thickness of 6000 cells/well (HeLa cells) in 100L medium. After several concentrations of crude extract add to the cells at 100L medium. Cells were brooded for twenty-four hours with test drugs. Every focus was tried in triplicate. Each concentration was tested in triplicate

The MTT is a test that estimates changes in shading for estimating the action of compound that lessens MTT to formazan giving a purple shading. Yellow MTT (3-(4, 5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide, a tetrazole) decreased to purple formazan in living cells[16]. After forty-eight hours hatching, 20 L (5 mg/mL) MTT component was added to each well and brooded for another four hours. At that point 200 L of DMSO was added to each well to dissolved the formazan precious stones. The plates were perused for optical density at 540 nm with reference 630 nm, utilizing a plate reader. By utilizing optical density, rate hindrance of was determined. The cytotoxicity measure was performed by utilizing the Sulforhodamine B colorimetric strategy to survey development restraint as per Vanicha and Kirtikara[17]. Quickly, till forty-eight hours treatment same as that of MTT strategy. Toward the finish of the uncovered time, cells in each well were fixed by expansion of 100 mL of cold [(four degree Celsius) 10%(w/v)] trichloroacetic acid (TCA) into the expansion medium. Each plate was brooded at 4°C for 1 h before delicately washed multiple times with Mili-Q water to evacuate TCA, the expansion medium and dead cells. Plates were permitted to dry in air and to each well were included 50 mL of 0.057% (v/v) SRB reagent in 1% acidic acide in deionized water and permitted to viewpoint till 30 min at room temperature. Toward the finish of the recoloring time frame, unbound SRB was expelled by washing multiple times with 1% of an acidic acid solvent. The plate was air-dried and 150 mL of 10 mM fluid Tris base buffer at pH 10.5 was added to each well to mix the cell-bound dye. The plate was then shaken for 15 to 30 min on a gyratory shaker and the optical density (OD) was read at 540 nm with standered 630 nm in a microplate reader; control group wells were utilized as blanks[18]. IC_{50} was calculated utilized prism graph pad-5 software.

Apoptosis studies

AOEB dual staining

AO/EB staining method recognizes live, early apoptotic, late apoptotic, and necrotic cells. 5 × 105 cellsl around was seeded in a 6-well plate the night prior to the treatment.

Cells were treated with chosen fractions, vehicle control group and positive control group at specific IC_{50} numbers for fort-eight hours at thirty-seven degree Celsius in CO_2 incubator apparatus. Aftertreatment, media comprising skimming cells and appended cells in 6 well plate were gathered into centrifuge tubes and centrifuged. Supernatant disposed of and cell pellets were resuspended in 1 mL of HBSS. 10 microlitre of EtBr solvent were included and kept in incubator for 10 min and cells were centrifuged at 1200 rpm for 4 min at four degree Celsius. Supernatant was depleted and pellets were unstuck. 20 microlitre of the cell suspensions was put on a slide and saw under the fluorescent magnifying/microscope instrument furnished with 450–490 nm excitation and 520 nm outflow wavelength (blue fil-ter)[19].

Nuclear staining with Hoechst-33342.

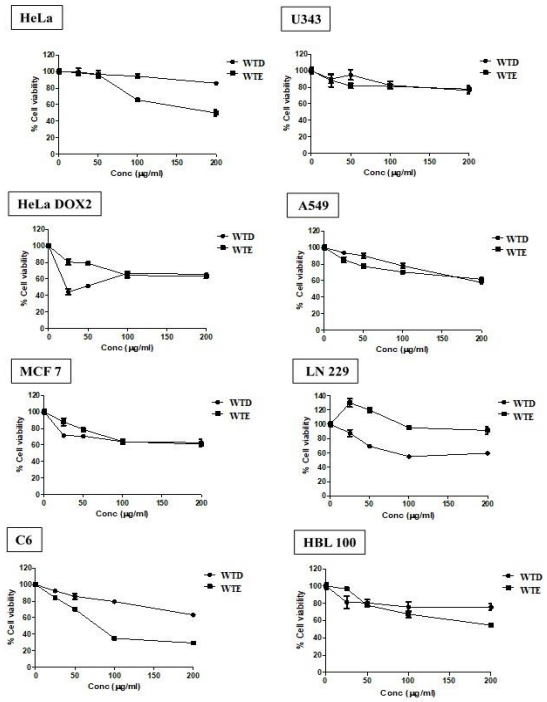

Figure1: The cytotoxicity of *W. tinctoria*. two fractions (i.e. WTD and WTE) against various cancer cell lines at 48 hrs. treatment by MTT method.

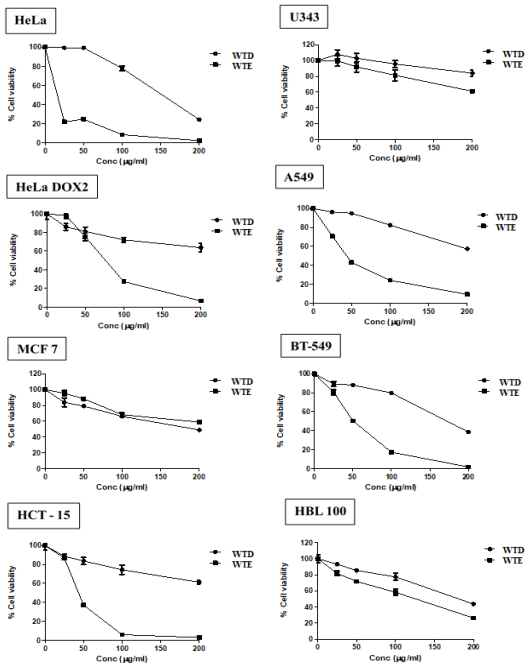

Figure 2: The cytotoxicity of *W. tinctoria*. two fractions (i.e. WTD and WTE) against various cancer cell lines at 48 hrs. treatment by SRB method.

Table No. 1: IC_{50} value of *W. tinctoria*. two fractions (i.e. WTD and WTE) in different cells by MTT and SRB method.

Type of cells	IC_{50} (µg/mL)			
	MTT Method		SRB Method	
	WTD	WTE	WTD	WTE
HeLa	773.6	186.3	150.03	12.75
HeLa DOX2	321.4	430.6	459.1	72.77
U343	917.7	3230	366.0	268.8
A549	256.4	334.1	237.3	39.20
MCF 7	461.6	313.7	200.7	251.9
BT-549	-	-	168.1	51.92
HCT 15	-	-	358.2	41.09
LN 229	234.8	384.7	-	-
C6	367.8	79.61	-	-
HBL100	-	-	181	125

The nuclear morphology of cells was investigated by utilizing cell penetrable DNA dye Hoechst33342. Cells with homogeneously recolored stained were viewed as feasible, though the nearness of chromatin buildup and additionally discontinuity was characteristic of apoptosis. The cells were treated with IC_{50} of the chose fractions and further brooded for forty-eight hours. Cells were centrifuged and supernatant disposed of. Cell pellets were resuspended in 1 mL of HBSS. 05 micolitre Hoechst-33342-dye solvent were included and kept in incubator for ten min and centrifuged at 1200 rpm for four min at four degree Celsius. Supernatant was depleted and pellets were resuspended. 20 microlitre of the cell suspensions was put on a slide and saw under the fluorescent magnifying lens outfitted with excitation wellspring of 350 nm and outflow at 450 nm (UV channel)[20].

Determination of DNA fragmentation.

The trademark stepping stool example of DNA breakage was investigated by gel electrophoresis. Malignancy cells were set in a 6-well plate at a centralization of 5 × 105 cell/mL. The cells were treated with various concentration of chose fractions with positive control group and were additionally brooded for twenty-four hours. The DNA was disengaged and electrophoretically examined on 1.5% agerose gel comprising 10L/mL ethidium bromide[21].

Alkaline comet assay.

After twenty-four hours culture commencement, the cells were treated for forty-eight hours with concentrations of drugs. The cells were eroded with HBSS and collected. The slides were secured with 1.5% typical liquefying point agarose, permitted to set at twenty-five-degree celsius and put away at four-degree celsius till one hour. An aliquot of the cell suspension was mixed of 0.75% low liquefying point agarose. This blend was quickly pipetted on to the

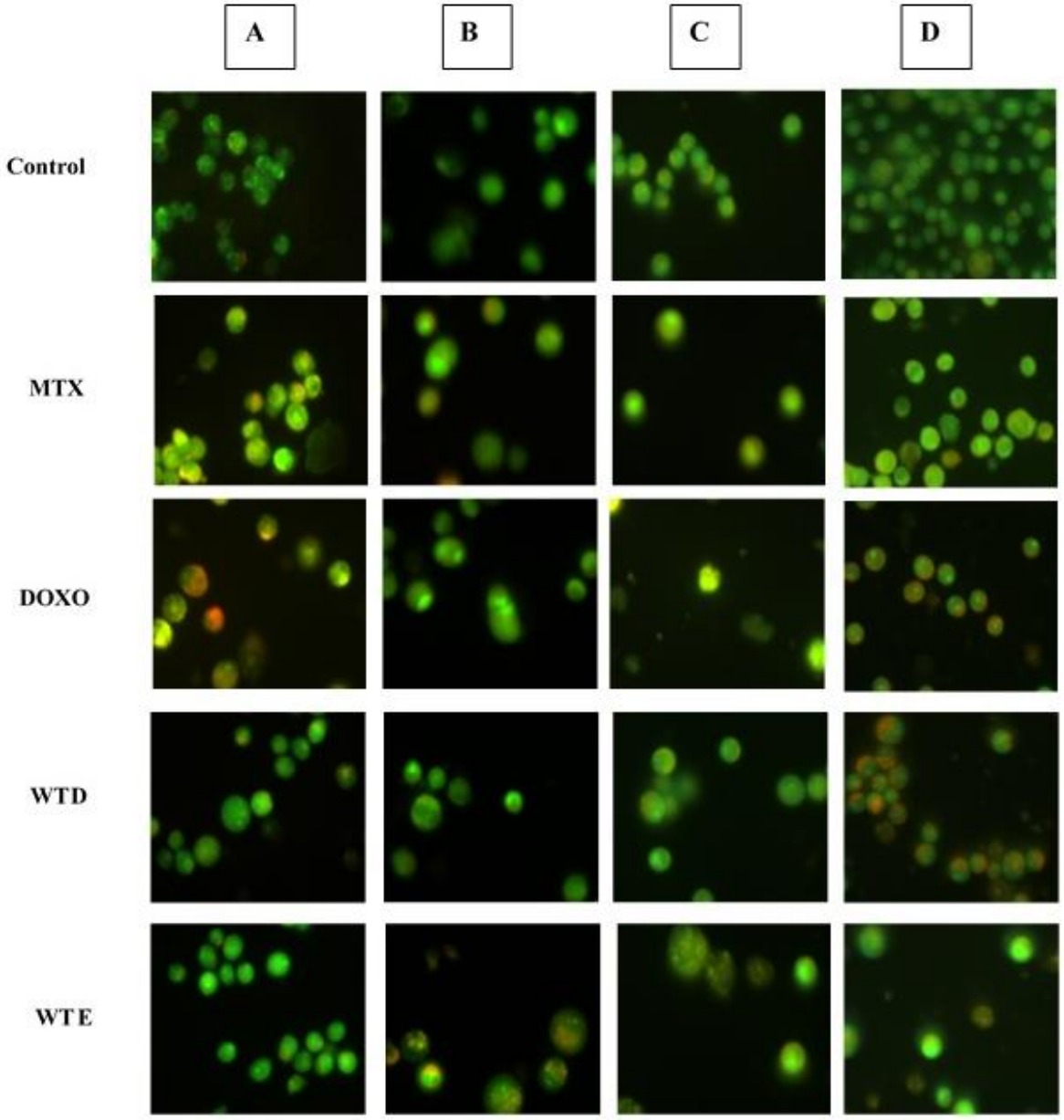

Figure 3: Acridine orange assay for the discrimination of apoptosis vs. necrotic cells after treatment of *W. tinctoria*. two fractions (i.e. WTD and WTE) on various cells by dual staining (A) Human Cervical carcinoma (HeLa), (B) Human Neuronal Glioblastoma (U343), (C) Lung adenocarcinoma (A549), (D) Human breast carcinoma (BT549)

agarose layer on the slide, tenderly spread by putting a coverslip on top and permitted to harden at four- degree celsius till five min. After evacuation of the coverslip, the slides were inundated in newly ready lysis solvent (2.5 M NaCl, 100 mM EDTA, 10mMTris, pH 10, with 1% TritonX-100 and 10% DMSO) for 12 hours at four-degree celsius. At that point, the slides were left in the electrophoresis solvent (1 m MEDTA and 300mM NaOH, pH 13 at four- degree celsius) for one hour to take into consideration DNA loosening up and articulation of alkali-labile damage before electrophoresis. Electrophoresis was done at 22 V and 300 mA for 20 min at four- degree celsius. Then, the slides were clened in neutralized buffer (0.4 M Tris/HCl, pH 7.5) and recolored with 80L of a watery solvent containing20 mg/mL ethidium bromide. Nucleoids were analyzed visually[22].

Cell cycle analysis.

So as to examine the connection between cell expansion hindrance and the acceptance of apoptosis, we chose to consider the subdiploid DNA contends as demonstrative of DNA fragmentation using apoptosis. DNA substance and cell cycle dispersion were surveyed utilizing PI staining. After sample treatment, both floating and adherent cells were reaped, cleaned with phosphate buffed saline (PBS) and fixed with overnight busing ethanol at twenty-degree celsius. Fixed cells were clened and resuspended in a buffer containing 50g/mL of RNAse A for three hours, included 25g/mL of Propidium iodide and investigated by flow cytometry. Propidium iodide was energized by a 488

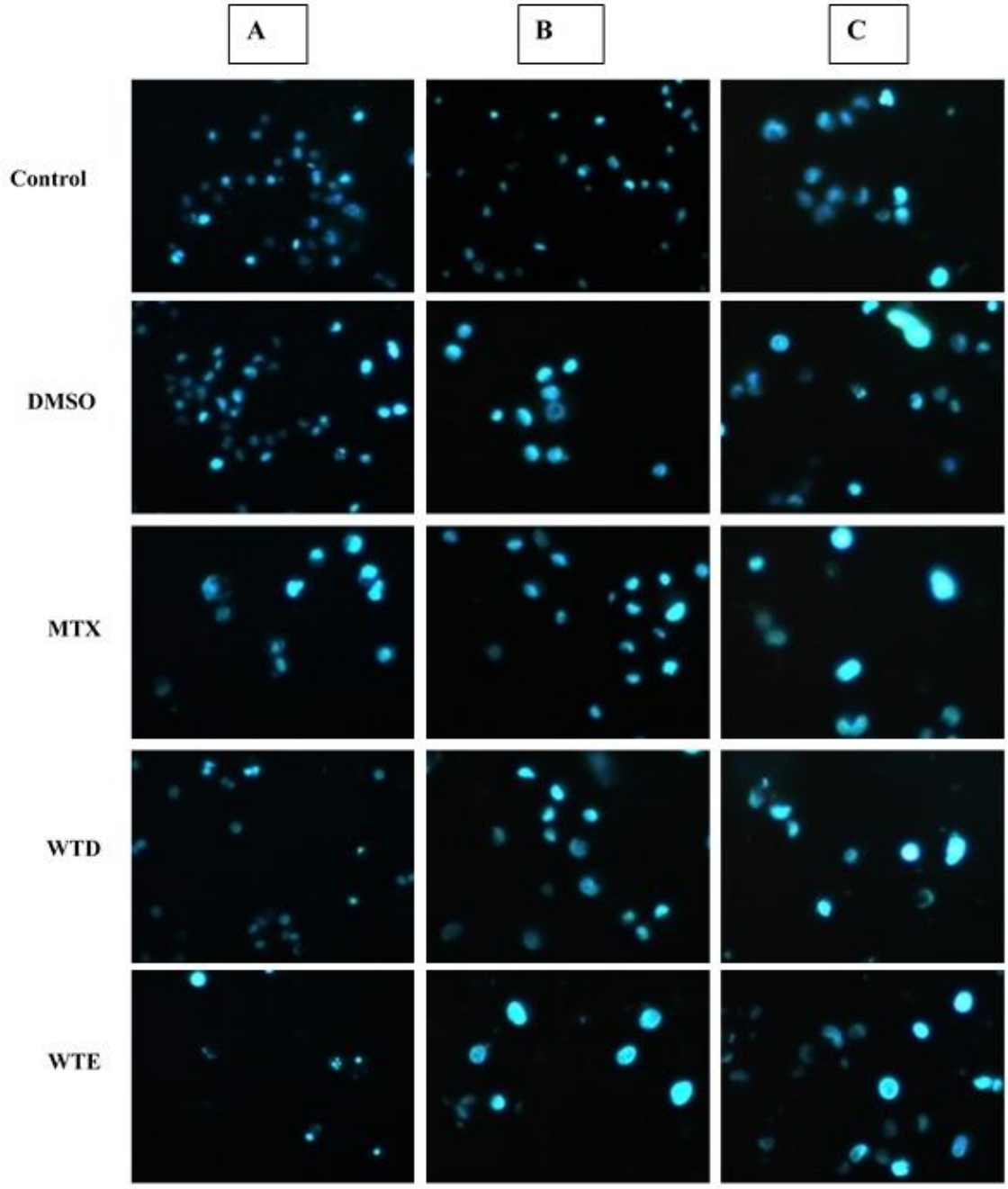

Figure 4: Hoechst-33342 DNA staining assay. A. Human cervical carcinoma (HeLa). B. Human neuronal glioblastoma (U343). C. Normal epithelial breast cells (HBL100).

nm laser and emanation was caught at FL-2 (570/20 BP channel). DNA content in various periods of the cell cycle were evaluated for both the control group and the treated cells group. Calculation of observation was done by Summit v4.3 programming[23, 24].

2.5.3. Genotoxic assessment

2.5.3.1. Micronucleus assay.

The micronucleus examine was carried out by Matsuoka et al.[25]. Around 1 × 106 cells/mL medium were uncovered and hatched. Complete media were treated as positive group and negative controls group individually. Toward the finish of hatching, the cells were collected by low centrifugation, treated with a hypotonic solvent of KCl (0.075 M) and fixed in methanol: acidic acid (3:1) for three to four hours. 2-3 drops of the fixed cell suspension were administered on to the outside of cold micro scale slides, air-dried and recolored with 3% Giemsa solvent in Sorenson phosphate buffer (pH 6.8) till five to seven min. The bi, tri or multi nuclei cell was seen in slides[26].

Statistical analysis

All the experimentations were individualistically carried out thrice with three replicated for each group. The

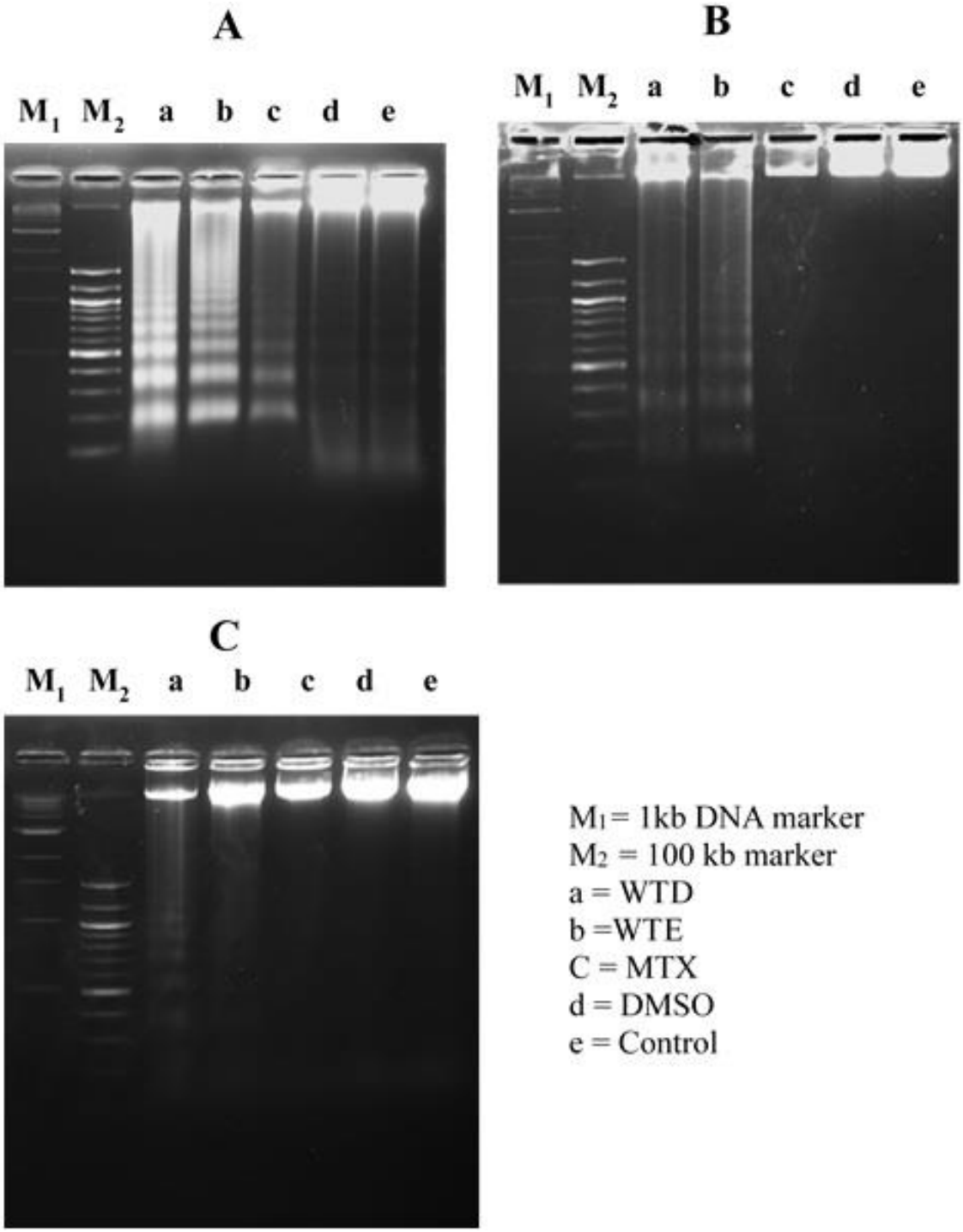

Figure 5: DNA fragmentation assay. A. HeLa (Human cervical carcinoma). B. A549 (human lung adenocarcinoma). C. U343 (human glioblastoma).

outcomes were stated as mean ± SEM and examined by ANOVA by Turkey test and $P < 0.05$, $P < 0.01$, $P < 0.001$ was considered statistically significant.

RESULTS AND DISCUSSION

As of late, the utilization of certain herbs has pulled in a lot of consideration as one of the elective malignancy treatments from the purpose of less poisonous quality and money saving advantages. Along these lines, an endeavor has been made to assess the anticancer movement of the plant alcoholic extract of *Wrightia tinctoria (Roxb.)*, which is generally utilized in ayurvedic system of drug for different purposes.

Cytotoxic effect of the Wrightia tinctoria (Roxb.) on human cancer cells

Table No. 2: Induction of DNA damage assessed by alkaline comet assay (% Tail DNA and olive tail moment) in HeLa and A549 cells treated with various concentrations of *W. tinctoria*.

Drugs	Types of cell lines			
	HeLa		A549	
	% Tail DNA	Olive tail moment	% Tail DNA	Olive tail moment
Untreated	4.26±2.73	5.37±2.64	3.65±2.51	2.35±2.46
DOXO	29.48±5.13	65.04±9.21	45.15±7.33	95.92±3.48
MTX	48.82±8.22	141.65±13.54	48.01±4.68	84.33±7.92
WTD	79.49±3.16	328.92±07.06	8.90±2.31	15.60±4.61
WTE	27.54±4.94	64.33±4.78	67.12±2.56	139.13±6.35

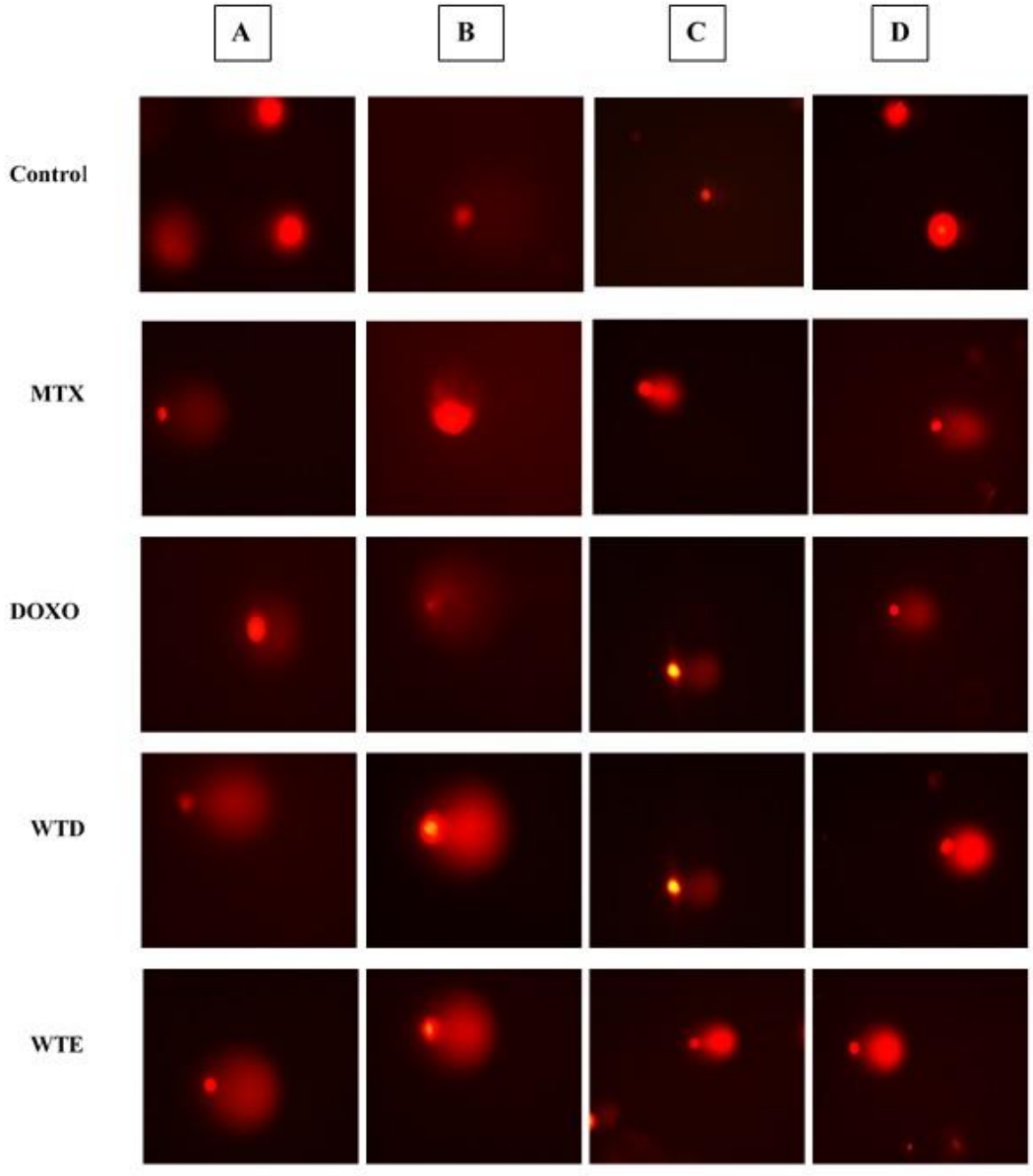

Figure 6: COMET assay after treatment with *W. tinctoria*. on different cells (A) Human cervical carcinoma (HeLa), (B) Human neuronal glioblastoma (U343), (C) Lung adenocarcinoma (A549), (D) Human breast carcinoma (BT549)

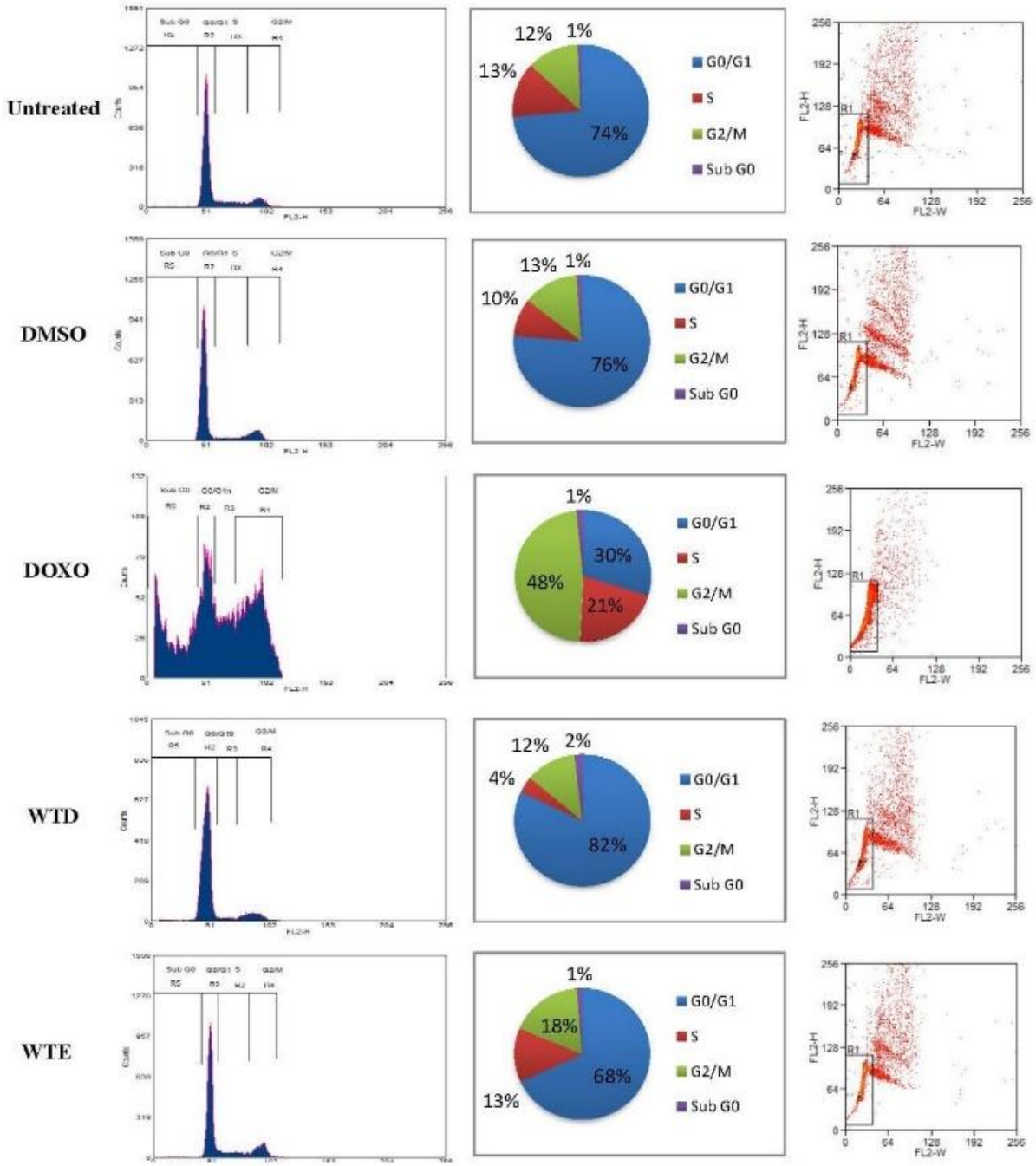

Figure 7: Cell cycle analysis by flow cytometry in Human cervical carcinoma (HeLa) cells after treatment with fractions of *W. tinctoria***.**

In screening HeLa cells were the most vulnerable to the treatment, while U343 were the least. Test is put together up with respect to decrease of yellow tetrazolium salt (MTT) by the reductase compound in metabolically dynamic cells to a dull blue formazan[27] [20]. These exercises were bolstered by Sulphorhodamine B measure. The inhibitory impact on cell reasonability was apparent after forty-eight hours of brooding and most cytotoxic portion (DCM and ethyl acetic acid derivation) was chosen for additionally assessed at different cells by deciding the IC_{50} esteem. In ethyl acetic acid derivation portion powerful cytotoxic IC_{50} qualities were found against human cervical carcinoma186.3 g/mL and 12.75 g/mL by MTT and SRB strategy individually. Be that as it may, critical cytotoxic action was discovered uniquely by SRB strategy (150.03 g/mL IC50value) in DCM fraction. IC_{50} values in Human adenocarcinoma alveolar basal epithelial cells 39.20 g/mL, Human bosom ductal carcinoma cells (BT-549) 51.92g/mL, Human colorectal adenocarcinoma (HCT-15) 41.09 g/mL by SRB technique were discovered powerful cytotoxic action on ethyl acetic acid fraction. MTT indicated noteworthy cytotoxic movement in glioma

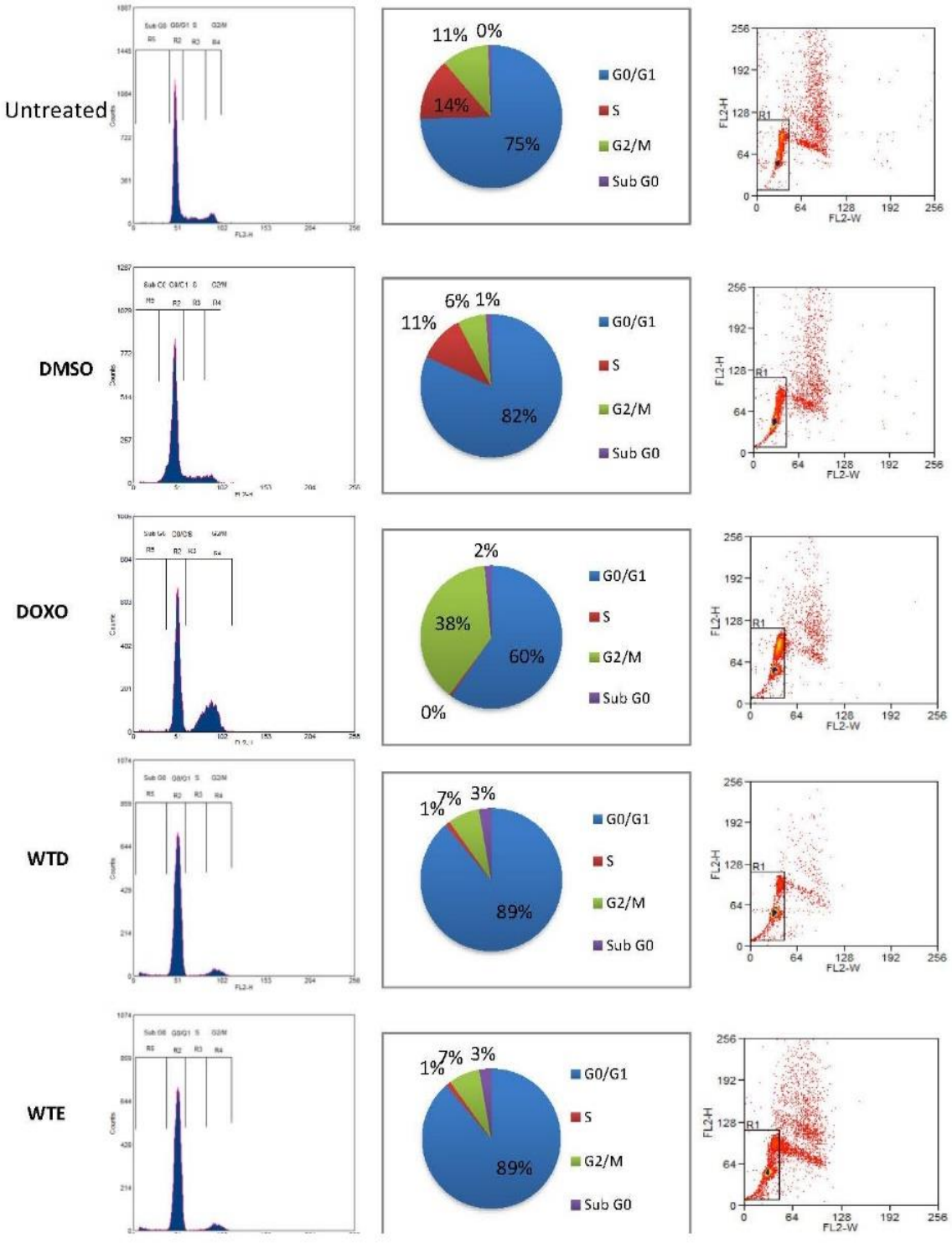

Figure 8: Cell cycle analysis by flow cytometry in Lung adenocarcinoma (A549) cells after treatment with fractions of *W. tinctoria*.

cells (C6) with IC_{50} value 79.61g/mL. Consequences demonstrated that ethyl acetate portion altogether diminished practicality of malignant growth cells in portion subordinate way (Fig. 1 & 2, Table 1).

Induction of apoptosis in cancer cells by the selected active fraction

Acceptance of apoptosis in malignant growth cells is one helpful procedure for anticancer medication

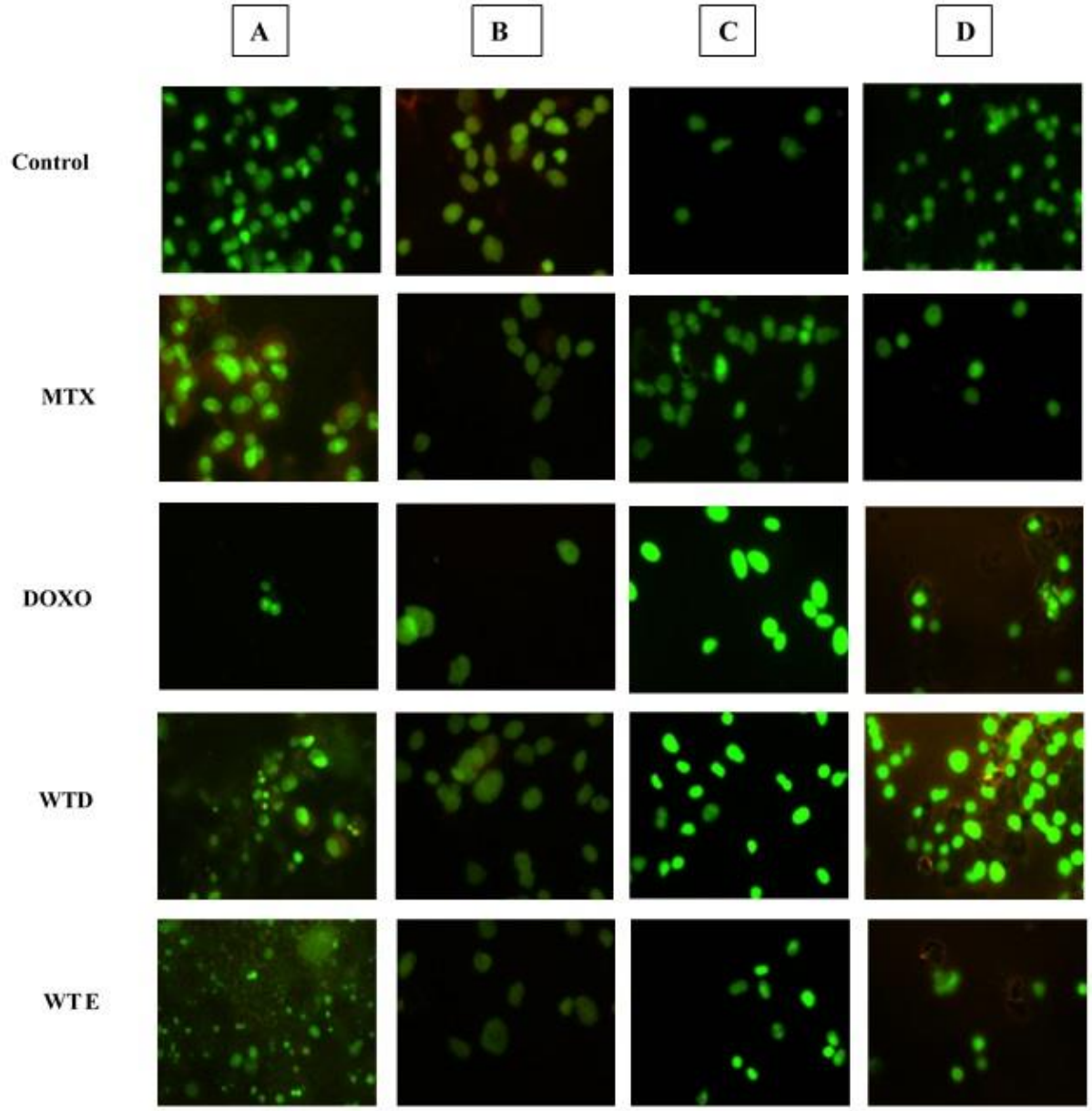

Figure 9: Micronuclei formation assay after treatment with fractions of *W. tinctoria*. on different cells (A) Human Cervical carcinoma (HeLa), (B) Human Neuronal Glioblastoma (U343), (C) Lung adenocarcinoma (A549), (D) Human breast carcinoma (BT549)

advancement[27]. In this regard, numerous phytoconstituents starting point have been tried for their ability to induce apoptosis[27, 28]. Following forty-eight hours hatching of malignant growth calls at different concentration brought about the presence of an enormous number of cells in the sub-G1phase (apoptotic cells). Besides, a decrease of cells in the G0/G1phase and cell capture in the S stage was watch dafter forty-eight hours treatment. Introduction of malignant growth cells to the chose fractions at various concentrations for forty-eight hours determines the typical morphological characteristics of apoptosis. Those progressions comprised cellular shrinkage, layer blebbing, nuclei of divided cells made up of apoptotic bodies, nuclear dissolution, cytoplasmatic film shrinkage and contact in adjacent cells. Comparative perceptions were made on cells treated with doxorubicin, though the introduction to the vehicle did not caused nuclear changes as affirmed by the nonappearance of staining.

AO/EB dual staining indicate that apoptosis induction

Acridine orange saturates all cells and makes the nucleus to seem green, although EtBr is taken up just by cells that have lost cytoplasmic film uprightness, and the nucleus of the red spot. Cell lines treated with positive control (methotrexate and doxorubicin), DCM fraction and ethyl acetate fractions with IC_{50} numbers. non-apoptotic live cells; although cell lines treated with positive control and DCM fraction and ethyl acetate fraction brought about a significant raised in the quantity of green spots (dense

DNA) nucleus demonstrating early apoptotic cells and green-red fragmented nucleus (condensed DNA) indicates late apoptotic cells. Treated cells demonstrated very consolidated chromosome with green fragment nuclei. (Fig. 3).

Hoechst-33342 staining reveals induction of apoptosis

To assess whether, the reduction in cellular susceptibility watched aftertreatment with DCM fraction and ethyl acetate fraction was because of apoptosis, the cells HeLa, HBL100 and U343 were examine with Hoechst-33342 day. This dye purifies the intensive chromatin of programmed cells more clearly than that which affects natural cell chromatin. Which connects with the nearness of cells with a run of the mill apoptotic atomic morphology (Nuclear contraction, intensification and fragmentation of DNA), was available in both fractions treated cells, however not in the untreated controls (Fig. 4).

DNA Fragmentation

DNA fragmentation is a trademark highlight of apoptosis. Expanded DNA fragmentation (DNA ladders) was evident in A549, U343 and HeLa cells after treatment with IC_{50} numbers of DCM fractions, ethyl acetic acid derivation division and methotrexate (positive control) for forty-eight hours. while treatment with DMSO (0.5%) (vehicle control) did not produce DNA fragment (Fig. 5).

Comet assay for apoptosis

The data indicate that there is a rise in the parameters of the comet, for example, % DNA in tail and olive tail moment (OTM) with specific fractions compared to the untreated control or solvent control (DMSO). Ethyl acetate fraction indicated most noteworthy raised in % tail DNA substance and OTM in, BT549, U343 and A549.Treatment with positive control (methotrexate) DCM fraction and ethyl acetate fraction demonstration noteworthy increment in comet parameters when contrasted with untreated cells. In HeLa and BT-549 cells, DCM fraction indicated most astounding increment in comet parameters (Fig. 6, Table 2).

Cell cycle analysis shows G1 arrest etc.

The impact of the both fractions on cell cycle movement on A549 and HeLa was dictated by flow cytometry method. DCM fraction and ethyl acetate fraction treatment demonstrated a huge increment in the level of G1-stage from seventy two percentage (untreated) of cells at the amount of IC_{50} proposing that DCM fractions the HeLa cells at G_0/G_1phase eighty two percentage and ethyl acetate fraction treatment indicated eighteen percentage G_2/M phase which is huge contrast with untreated cells twelve percentage. in A549 cells same as HeLa DCM fraction altogether expands the level of G_0/G_1phase eighty-nine percentage relative to untreated cells seventy-five percentage and ethyl acetate fraction raised the amount in sub G_0 seven percentage and G_2/M twenty on percentage than untreated cells sub G_0 nil and G_2/M fourteen percentage. Doxorubicin was taken as positive control and DMSO (0.5%) as vehicle control. Doxorubicin demonstrated totally G2arrest (Figs. 7 and 8).

Active fractions induce genotoxicity in cancer cells

Micronuclei development is a sign of possessions of medication instigated genotoxicity. Consequences demonstrate that DCM fraction and ethyl acetate were initiate to from micronuclei in all investigate cell lines. This shows capacity of fractions to cause chromosomal harm and genome variability in malignant growth cells (Fig. 9).

CONCLUSION

Alcoholic extract fractions of *W. tinctoria.* was examined for their in vivo anticancer action. In which two fraction DCM and ethyl acetate was discovered most dynamic in cytotoxic movement. We might want to reason that; the present investigation features the anticancer and cytotoxic capability of alcoholic extract fractions of *W. tinctoria*. These outcomes recommend that the cytotoxic action of this plant has been because of its apoptosis instigating assets. This was proved by interruption of mitochondrial layer potential, DNA fragmentation, externalized phosphatidyl serine and collection of sub-G_0 and G_1phase.

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