ORIGINAL ARTICLE

Flow cytometry analysis of *Dooshivishari agada*-induced cell cycle arrest in human T-cell acute lymphocytic leukemia (Jurkat) cell lines

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Abstract

Introduction: *Dooshivishari agada* is a herbo-mineral formulation that has been widely prescribed in the treatment of *~Dooshivisha* (cumulative poison), its complications, and also in the management of various other types of poisoning conditions. *~Dooshivisha* (cumulative poison) is a unique type of poisoning explained in *Ayurveda* classics, which is caused due to the cumulative deposition of various toxins in small quantities for a long period. This is more evident in the present era of modernization and urbanization. These deposited toxins are not fatal but cause diseases such as cancer, oxidative stress, and Parkinsonism. Leukemia is a type of cancer that has causative factors such as radiation, pesticides, and chemicals. The causative factor and pathology of leukemia are similar to *~Dooshivisha* (cumulative poison). Cell cycle analysis helps to assess the cytotoxic potentials of different components by assessing the normal cancer cell cycle arrest. The present study was designed to elucidate the cell cycle arrest induction by methanolic extract of *D. agada*, in the human T-cell acute lymphocytic leukemia (JURKAT) cell line. **Materials and Methods:** Cells were incubated with different concentrations of methanolic extract of *D. agada*, and the percentage of cells in various stages of the cell cycle in compounds treated and untreated populations was determined using FACS. **Results:** The treatment of cells at the concentrations of 80 µg/ml and 160 µg/mL of sample *D. agada* has shown G2M arrest from 5.58% (control) to 1.47% and 12.31%, respectively. S-phase arrest was found to be 13.54% and 15.51% at the same concentrations. **Discussion and Conclusion:** This laboratory study demonstrated the cytotoxic effect of the sample methanolic extract of *D. agada* through cell cycle arrest.

Key words: Cell cycle, *Dooshivisha*, *Dooshivishari agada*, Jurkat, leukemia

INTRODUCTION

Cancer is one of the most challenging clinical problems worldwide and leads to 21% of all mortality in developed countries and 9.5% of all mortality in the developing countries. According to the WHO, by 2050, it is predicted that approximately 27 million new cases of cancer and 17.5 million deaths from cancer annually.[1] Leukemia is the 11th most common cancer type worldwide.[2] Although the exact cause for cancer is not known, the increased usage of many toxic substances in day-to-day life leads to the deposition of small quantities in the body. After sometime when the toxin load gets increased and on getting favorable environment, it causes distressing conditions such as cancer, Alzheimer's disease, and renal and hepatic disease. Leukemia is a type of cancer that mainly affects white blood cells. The main causes are exposure to radiation, bacteria, virus, pesticides, food preservatives, etc.[3] The etiology and pathology of cancer and leukemia simulate with the *~Dooshivisha* (cumulative poison), which is a very unique concept of *Ayurveda*. *~Dooshivisha* (cumulative poison) is a transformable state of *~visha* (poison) which is having low potency and stays in the body for a long duration due to *~avarana* (enveloping) by *~Kapha*. Hence, it does not cause sudden death but produces

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illness related to the ~*dhatu* (tissue) where the toxin got deposited.[4,5] If the toxin is deposited in white blood cells, it produces diseases such as leukemia. The treatment of various types of cancer involves the use of chemotherapy and radiotherapy. These treatments gradually cause damage to normal cells, and resistance to targeted cells reduces the success rate of these therapies.[6] *Dooshivishari agada* is a herbo-mineral formulation explained in the context of the management of *Dooshivisha* (cumulative poison).[4]

Reproduction of cells requires cell division, with the production of two daughter cells with the same DNA content as the parent. The cycle of increase in components (growth) and division, followed by growth and division of these daughter cells, etc., is called the cell cycle. The two most obvious features of the cell cycle are the synthesis and duplication of nuclear DNA before division and the process of cellular division itself mitosis. These two components of the cell cycle are usually indicated in shorthand as the "S phase" and "mitosis" or "M."

When the S phase and M phase of the cell cycle were originally described, it was observed that there was a temporal delay or gap between mitosis and the onset of DNA synthesis, and another gap between the completion of DNA synthesis and the onset of mitosis. These gaps were termed G1 and G2, respectively. The cycle of G1 → S → G2 → M → G1, etc., is shown schematically in Figure 1.

One of the earliest applications of flow cytometry was the measurement of DNA content in cells. This analysis is based on the ability to stain the cellular DNA in a stoichiometric manner. The location to which these dyes bind on the DNA molecule varies with the type of dye used. The most common DNA binding dye in use today is the blue excited dye propidium iodide (PI). PI is an intercalating dye which binds to DNA and double-stranded RNA (and is thus almost always used in conjunction with RNaseA to remove RNA). When diploid cells which have been stained with a dye that stoichiometrically binds to DNA are analyzed by flow cytometry, a "narrow" distribution of fluorescent intensities is obtained.[7,8]

Objectives

The objectives of the study were to elucidate the cell cycle arrest induction by methanolic extract of *D. agada*, in the human T-cell acute lymphocytic leukemia (JURKAT) cell line.

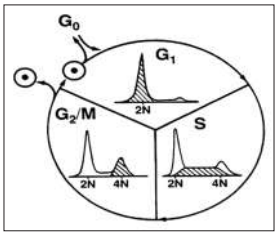


Figure 1: A schematic of the cell cycle, showing flow cytometric components of each phase

MATERIALS AND METHODS

Materials

All the raw materials and reagents are collected from the local market of Mysore.

Preparation of *D. agada*

Twelve ingredients of *D. agada, that is, Pippali (Piper longum), Dhyamaka (Cymbopogon martini), Jatamansi (Nardostachys jatamansi), Lodra (Symplocos racemosa), Ela (Elettaria cardamomum), Suvarchika (Tribulus terrestris), Kutannata (Oroxylum indicum), Nata (Valeriana wallichi), Kushta (Saussurea lappa), Yashtimadhu (Glycyrrhiza glabra), Chandana (Santalum album), and Gairika* (Red-ochre)[4] are collected from the local market, Hassan. A 10 g of each ingredient is taken, powdered separately, and mixed to a homogeneous mixture.

Methanolic Extract of *D. agada* Preparation

A 20 g of the prepared *D. agada* powder mixture is dissolved in 100 ml of methanol in a beaker. It is kept in a hot water bath at 50° C and incubated for 4 h. After the incubation period, it is filtered through a Whatman filter paper. The obtained filtrate is dried at 50° C few hours until the extract got completely dried and turned into semisolid form. It is used for further study.

Reagents Used

Propidium iodide

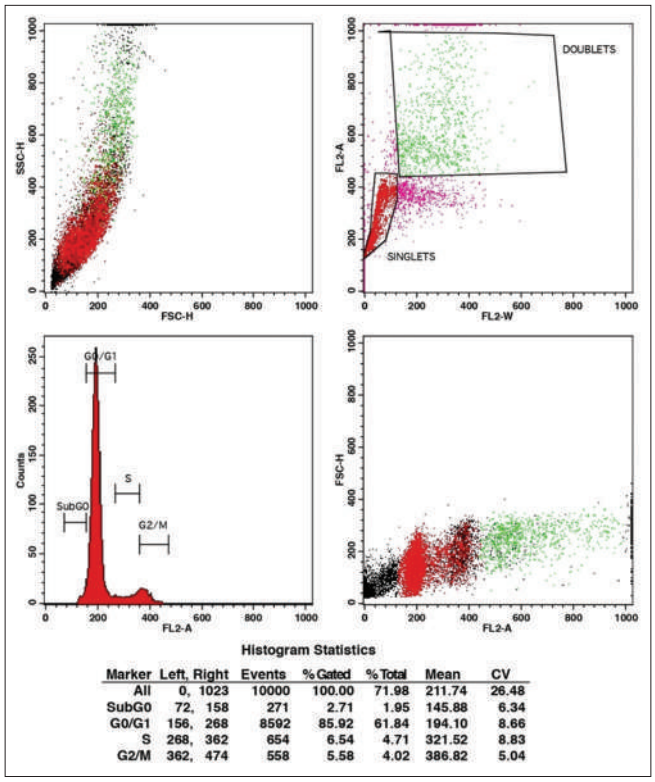
Cat # P4864, Sigma; stock 1 mg/ml, working solution is 0.05 mg/ml.

RNase A

Cat # 109169, Boehringer Mannheim GmbH; stock 5 mg/ml, working solution is 0.05 mg/ml.

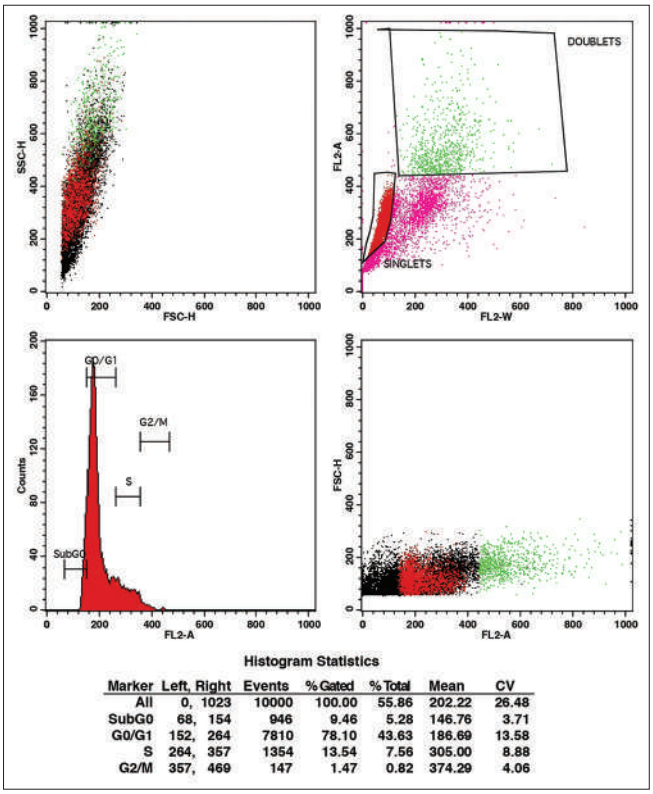
Procedure

1×10^6 cells were seeded and cultured for 24 h in a 6-well plate containing 2 ml of media. Cells were then treated with desired concentrations of given samples which were prepared in media and incubated for another 24 h. Cells were then harvested and centrifuged at 2000 rpm for 5 min at room temperature and supernatant was discarded carefully retaining the cell pellet. Cell pellet was washed by resuspending in 2 mL of 1X PBS. The washing was repeated another time with the



Marker	Left, Right	Events	% Gated	% Total	Mean	CV
All	0, 1023	10000	100.00	71.98	211.74	26.48
SubG0	72, 158	271	2.71	1.95	145.88	6.34
G0/G1	156, 268	8592	85.92	61.84	194.10	8.66
S	268, 362	654	6.54	4.71	321.52	8.83
G2/M	362, 474	558	5.58	4.02	386.82	5.04

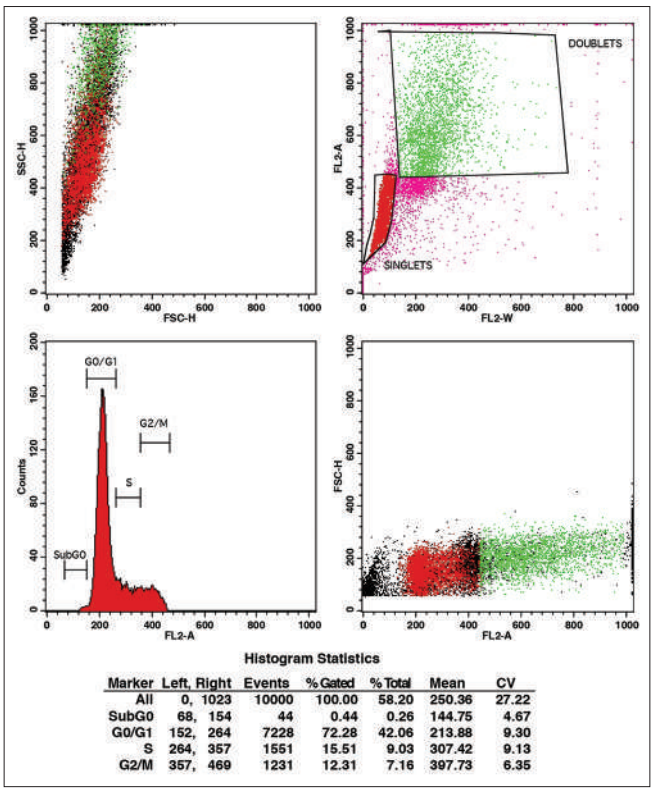
Figure 2: Flow cytometry control plots of JURKAT cells



Marker	Left, Right	Events	% Gated	% Total	Mean	CV
All	0, 1023	10000	100.00	55.86	202.22	26.48
SubG0	68, 154	946	9.46	5.28	146.76	3.71
G0/G1	152, 264	7810	78.10	43.63	186.69	13.58
S	264, 357	1354	13.54	7.56	305.00	8.88
G2/M	357, 469	147	1.47	0.82	374.29	4.06

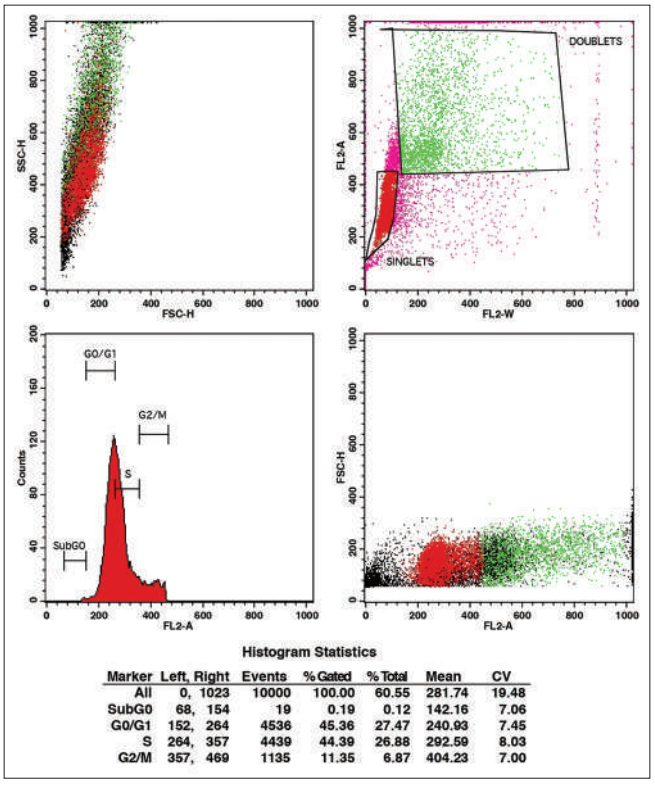
Figure 3: Flow cytometry plots of Jurkat cells treated with sample *Dooshivishari agada* at 80 µg/ml

same conditions. Supernatant was discarded retaining the pellet. Cells were fixed by resuspending in 300 µl of sheath fluid followed by addition of 1 mL of chilled 70% EtOH drop



Marker	Left, Right	Events	% Gated	% Total	Mean	CV
All	0, 1023	10000	100.00	58.20	250.36	27.22
SubG0	68, 154	44	0.44	0.26	144.75	4.67
G0/G1	152, 264	7228	72.28	42.06	213.88	9.30
S	264, 357	1551	15.51	9.03	307.42	9.13
G2/M	357, 469	1231	12.31	7.16	397.73	6.35

Figure 4: Flow cytometry plots of JURKAT cells treated with sample *Dooshivishari agada* at 160 µg/ml



Marker	Left, Right	Events	% Gated	% Total	Mean	CV
All	0, 1023	10000	100.00	60.55	281.74	19.48
SubG0	68, 154	19	0.19	0.12	142.16	7.06
G0/G1	152, 264	4536	45.36	27.47	240.93	7.45
S	264, 357	4439	44.39	26.88	292.59	8.03
G2/M	357, 469	1135	11.35	6.87	404.23	7.00

Figure 5: Flow cytometry plots of Jurkat cells treated with standard doxorubicin at 25 µM

by drop with continuous gentle shaking and another 1 mL of chilled 70% EtOH added at once. The cells were then stored at

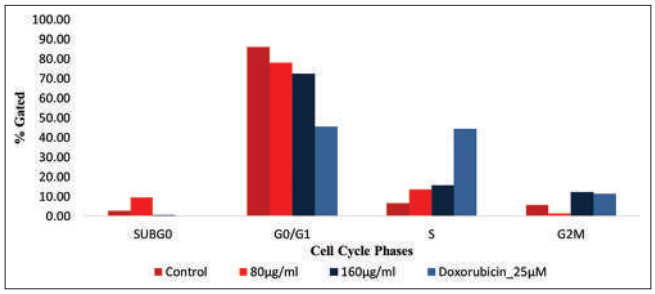


Figure 6: Flow cytometry analysis of cell cycle arrest in Jurkat cells

Table 1 : Flow cytometry analysis of cell cycle arrest in Jurkat cells

Sample	FACS analysis of cell cycle arrest in Jurkat cells			
	SUBG0	G0/G1	S	G2M
Control	2.71	85.92	6.54	5.58
80 µg/ml	9.46	78.10	13.54	1.47
160 µg/ml	0.44	72.28	15.51	12.31
Doxorubicin_25 µM	0.19	45.36	44.39	11.35

4° C for or overnight. Post-fixing, the cells were centrifuged at 2000 rpm for 5 min. The cell pellet was washed twice with 2 ml of cold 1X PBS. Cell pellet was then resuspended in 450 µl of sheath fluid containing 0.05 mg/ml PI and 0.05 mg/ml RNase A and incubated for 15 min in dark. The percentage of cells in various stages of cell cycle in compounds treated and untreated populations was determined using FACS Caliber.[7-9]

RESULTS

In control, out of 10,000 cells, 271 (2.71%) cells were found in sub G0 phase, 8592 (85.92%) cells were found in G0/G1 phase, 654 (6.54%) cells were found in the S phase, and about 558 (5.58%) cells were found at G2/M phase (Figure 2).

In JURKAT cells treated with *D. agada* at 80 µg/ml, out of 10,000 cells, 946 (9.46%) cells were found in sub G0 phase, 7810 (78.10%) cells were found in G0/G1 phase, 1354 (13.54%) cells found at S phase, and about 147 (1.47%) cells were found at G2/M phase (Figure 3).

In JURKAT cells treated with *D. agada* at 160 µg/ml, out of 10,000 cells, 44 (0.44%) cells were found in sub G0 phase, 7228 (72.28%) cells were found in G0/G1 phase, 1551 (15.51%) cells found at S phase, and about 1231 (12.31%) cells were found at G2/M phase (Figure 4).

In Jurkat cells treated with doxorubicin at 25 µM, out of 10,000 cells, 19 (0.19%) cells were found in sub G0 phase, 4536 (45.36%) cells were found in G0/G1 phase, 4439 (44.39%) cells found at S phase, and about 1135 (11.35%) cells were found at G2/M phase (Figure 5).

The treatment of cells at the concentrations of 80 µg/ml and 160 µg/mL of sample *D. agada* has shown G2M arrest from 5.58% (control) to 1.47% and 12.31%, respectively. S-phase arrest was found to be 13.54% and 15.51% at the same concentrations is summarized in Table 1 and same is represented as graph in Figure 6.

DISCUSSION

D. agada is widely practiced for the management of various conditions related to *~Dooshivisha* (cumulative poison). Methanolic extract of the sample is obtained and assessed for the cell cycle arrest of JURKAT cells. The ingredients of *D. agada* possess various activities such as antioxidant, anti-inflammatory, cytotoxic, anti-tumor, and anti-proliferative activity.[10] The development and progression of cancer is associated with disorders in the regulation of the cell cycle.[11] The cell cycle checkpoints are frequently deranged in cancer, causing them to enter cellular division, when conditions are not ideal and allow them to proliferate at an uncontrolled rate.[11,12] Subsequent experiments addressed the issue whether methanolic extract of *D. agada* pre-treatment perturbs the cell cycle progression in human breast cancer cells. Flow cytometric analysis of cell cycle distribution after PI staining of DNA clearly indicated G2M arrest and S-phase arrest in JURKAT cells. *~Dooshivisha* (cumulative poison) is a type of poison having *~Kaphapradhanata* (*Kapha* pre-dominancy) and *~avarana* (covering) property. The ingredients of *D. agada* are having *~Kapha-Vatahara* (reduces *kapha* and *vata*) and *~tridoshahara* (reduces all three humors) properties which helps to maintain the *doshas* in equilibrium state and hence reduces the effect of *~Dooshivisha* (Cumulative poison) in the body.[13]

CONCLUSION

The present study reveals potential mechanisms for the cytotoxic effects of *D. agada* on JURKAT cells. The anticancer effects of *D. agada* on JURKAT cells are mediated through cell cycle arrest at G2M phase and S phase. *D. agada* could be effective for the prevention and treatment of human T-cell acute lymphocytic leukemia cancer. Further investigation of its activity in animal models will help to better elucidate the role for this agent in the chemoprevention of leukemia.

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