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FLOW CYTOMETRY PROFILE OF DOOSHIVISHARI AGADA METHANOLIC EXTRACT INDUCED APOPTOSIS IN JURKAT (HUMAN T CELL ACUTE LYMPHOCYTIC LEUKEMIA) CELL LINES

Ayurveda

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ABSTRACT

INTRODUCTION: In the present era of modernization and urbanization people are exposed to many toxins in small quantities in their day-to-day life. They get heap up in the body without producing any immediate features. On getting favorable conditions tenacious diseases like alzheimer's disease, cancer, parkinsonism, etc. are produced in the tissue where the toxin got deposited, same unique concept is explained in Ayurveda as *Dooshivisha* (cumulative toxin). The etiology and pathology of *dooshivisha* (cumulative toxin) are similar to the etiology and pathology of cancer. *Dooshivishari agada* is explained as the formulation of choice for the management of *dooshivisha* (cumulative toxin). Apoptosis is a method to assess the cytotoxic potentials of different drugs. **OBJECTIVES:** The present study was designed to elucidate the apoptosis induction by methanolic extract of *Dooshivishari 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 *Dooshivishari agada*, and cell morphologic changes and apoptosis were determined by the normal inverted microscope, Annexin V, and propidium iodide (PI), followed by flow cytometric analysis, respectively. **RESULTS:** Sample *Dooshivishari agada* at 80µg/ml and 160µg/ml treatment have induced early and late apoptosis in JURKAT with 13.65%, 25.59%, and 2.36%, 10.24% apoptotic cells respectively, 1.88% and 4.08% necrotic cells were found when compared to control cells with 3.64%. **CONCLUSIONS:** Our preclinical study demonstrated a JURKAT cell line to be highly sensitive to *Dooshivishari agada* methanolic extract-induced apoptotic cell death.

KEYWORDS

Apoptosis, Dooshivishari agada, Flow cytometry, Leukemia

INTRODUCTION:

Dooshivisha (cumulative toxin) is the unique concept of the Ayurveda explained by *Acharyas* in the classics of Ayurveda. The incidence of *dooshivisha janya* (cumulative toxin caused) disease is increasing because of the accumulation of the small number of toxins in day-to-day life. These small amounts of toxins get heap up in the body and on getting favorable environment over a period, people will get affected with different tenacious diseases such as Alzheimer's diseases, Cancer,Parkinsonism, etc. Continuous exposure to carcinogenic agents such as radiation, chemical carcinogens like benzene, formaldehyde, asbestos, pesticides, and other agricultural chemicals, addiction of tobacco and most of the preservatives, infections viz RNA virus, some genetic factors causes leukemia. By this, we can understand that leukemia and *dooshivisha* (cumulative toxin) have similar etiology and pathology[1].

Leukemias are a group of disorders characterized by the accumulation of malignant white blood cells in the bone marrow and blood, in which abnormal growth of the white blood cells occurs due to persistent exposure to carcinogens. It is primarily two types Myeloid and Lymphoid, further subdivided into Acute and Chronic[2]. It is the 11th most common cancer worldwide[3]. As per Globocan 2020 statistics in India leukemia is the 7th most common type of cancer with an incidence rate of 48,419 and 35,392 deaths[4]. The mainstream treatment strategy for advanced leukemia is chemotherapy, radiation therapy, and stem cell transplant. Despite the initial success of chemotherapy, it affects the bone marrow's ability to produce adequate numbers of blood cells in later stage[5]. Thus, there is a potential role for newer chemopreventive compounds that can prevent or slow the progression of cancer.

As leukemia is correlated with *dooshivisha* (cumulative toxin), *Dooshivishari agada* may prove beneficial in the treatment of leukemia. *Dooshivishari agada* is a herbo mineral formulation explained by *Acharya Vagbhata* in *Ashtanga hrudaya* for the management of *dooshivisha* (cumulative toxin) [6]. It contains twelve ingredients where all these ingredients individually possess anticancer and cytotoxic activity through various in-vitro studies.[7] The present study aimed to assess the combined effects of ingredients *Dooshivishari agada* to have apoptotic activity, on human T cell acute lymphocytic leukemia (JURKAT) cells.

MATERIALS AND METHODS:

Materials:

The ingredients of *Dooshivishari agada* viz. *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)[6] are collected from the local market. 10gm of each ingredient is taken, powdered separately and mixed to a homogeneous mixture.

Preparation of Methanolic Extract of *Dooshivishari agada:*

To obtain a methanolic extract of *Dooshivishari agada*, 20 gm of dried powder of *dooshivishari agada* dissolved in 100ml of methanol in a beaker and kept on hot water bath at 50º C for 4 hours. After the incubation period, the extract was filtered with Whatmann filter paper and the filtrate was collected in a beaker. The residue present over the filter paper was discarded and the filtrate was kept at 50ºC for a few hours until the extract got completely dried and turned into semisolid form. It is used for further study.

Apoptosis study

Apoptosis is a cell death process characterized by morphological and biochemical features occurring at different stages. Different changes on the surface of apoptotic cells such as the expression of thrombospondin binding sites, loss of sialic acid residues, and exposure of a phospholipid like phosphatidylserine (PS) occur.

Phospholipids are asymmetrically distributed between inner and outer leaflets of the plasma membrane with phosphatidylcholine and sphingomyelin exposed on the external leaflet of the lipid bilayer, and phosphatidylserine predominantly observed on the inner surface facing the cytosol. Exposure of PS on the external surface of the cell membrane has been reported for activated platelets and senescent erythrocytes. Recently, it was shown that cells undergoing apoptosis break up the phospholipid asymmetry of their plasma membrane and expose PS which is translocated to the outer layer of the membrane. This occurs in the early phases of apoptotic cell death during which the cell membrane remains intact. This PS exposure may represent a hallmark (early and widespread) in detecting dying cells. Annexin V,

belonging to a recently discovered family of proteins, the annexins, with anticoagulant properties has proven to be a useful tool in detecting apoptotic cells since it preferentially binds to negatively charged phospholipids like PS in the presence of Ca2+ and shows minimal binding to phosphatidylcholine and sphingomyelin.

Changes in PS asymmetry, which is analyzed by measuring Annexin V binding to the cell membrane, were detected before morphological changes associated with apoptosis have occurred and before membrane integrity has been lost. By conjugating FITC (fluorescein isothiocyanate) to Annexin V it is possible to identify and quantitate apoptotic cells on a single cell basis by flow cytometry. Staining cells simultaneously with FITC-Annexin V (green fluorescence) and the propidium iodide (red fluorescence) allows the discrimination of intact cells, early apoptotic and late apoptotic or necrotic cells. [8]

Procedure:

Human T cell acute lymphocytic leukemia (JURKAT) cells are used in this study. The day before induction of apoptosis, plated 1 X 10^6 cells per well for a 6-well plate using DMEM cell culture medium. After ~18 hours, the wells for floating (dead) cells and removed by pipette. Replaced with the new culture medium to the original volume. JURKAT cells are treated with methanolic extract of *Dooshivishari agada* at 80µg/ml and 160µg/ml concentrations, and Doxorubicin_25uM as the standard control. The untreated cells served as the negative control. Treated cells to induce apoptosis with desired concentrations, and incubate for 24 hours. Later, the collected cell culture medium was transformed into 15-mL tubes. Using policeman, the cells were detached from the dish and added 1 mL of medium to each well, and transferred the contents were to the 15-mL tubes. Centrifuged and discarded the supernatant. Washed the cells twice with cold PBS (phosphate-buffered saline) and then resuspend cells in 1 mL 1X Binding Buffer at a concentration of ~1 x 10^6 cells/mL. 500µL of cell suspension is aliquoted and 10µL of PI (propidium iodide) and 5 µL Annexin V is added. The suspension is incubated for 15 minutes at room temperature in the dark. Post incubation, the cells were analyzed by flow cytometer as soon as possible (within 1 hour). The JURKAT cells were gated separately according to their granularity and size on forward scatter (FSC) versus side scatter (SSC) plots. Early and late apoptosis was evaluated on fluorescence 2 (FL2 for propidium iodide) versus fluorescence 1 (FL1 for Annexin) plots. Cells stained with only Annexin V were evaluated as being in early apoptosis; cells stained with both Annexin V and propidium iodide (PI) were evaluated as being in late apoptosis or a necrotic stage.

RESULTS:

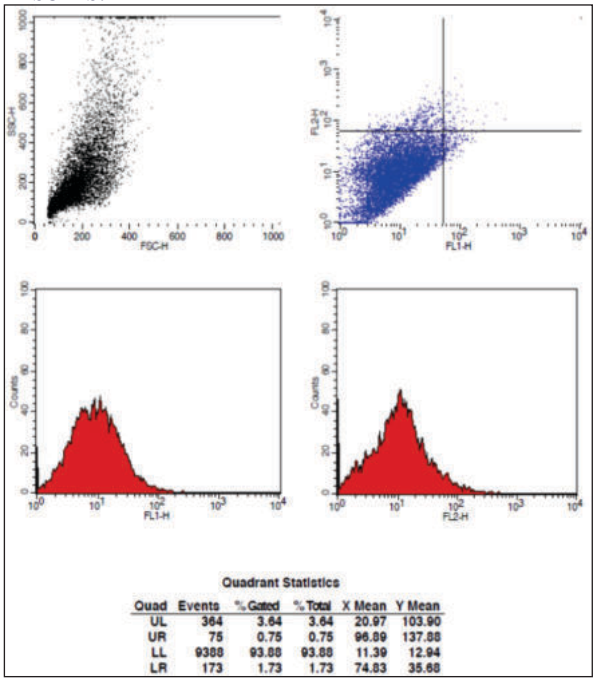

Figure 1: JURKAT untreated cells

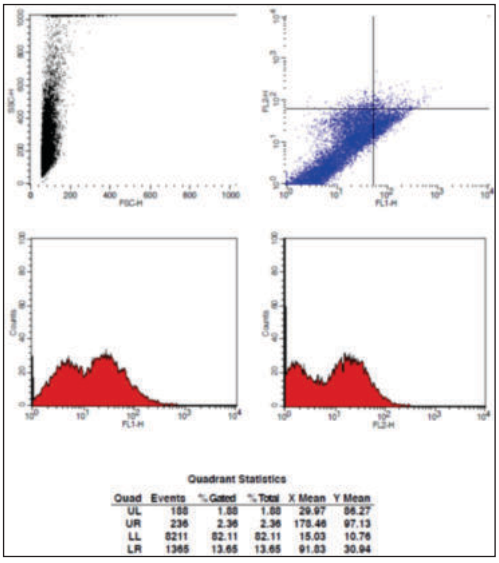

Figure 2: JURKAT cells treated with Sample *Dooshivishari agada* at 80µg/ml

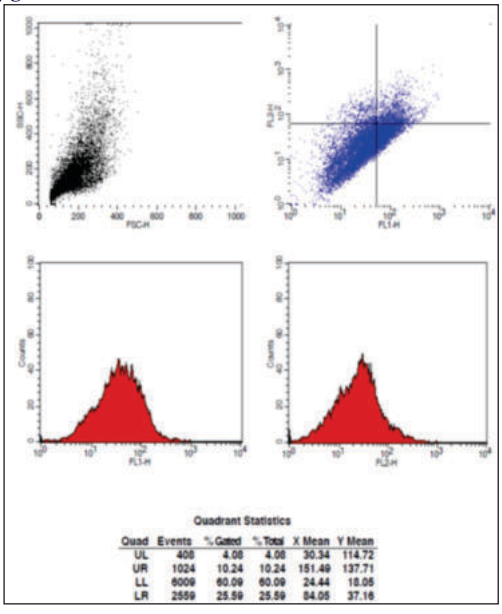

Figure 3: JURKAT cells treated with Sample *Dooshivishari agada* at 160µg/ml

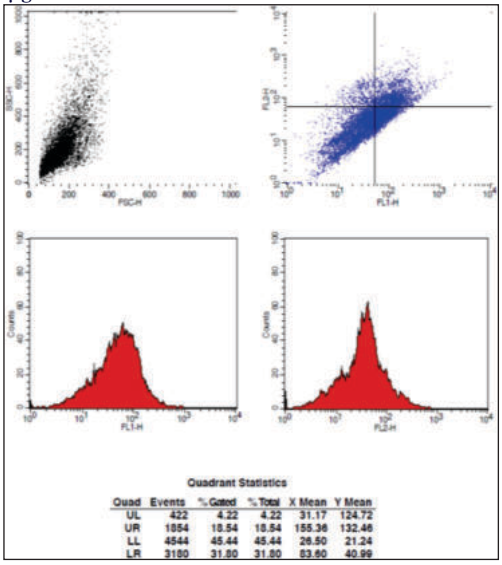

Figure 4: JURKAT cells treated with Standard Doxorubicin at 25µM

Table 1: Flow cytometry analysis of Apoptosis detection of JURKAT cells

	FACS analysis of Apoptosis detection in JURKAT cells			
Sample	**Viable cells**	**Early Apoptotic**	**Late Apoptotic**	**Necrotic cells**
Control	93.88	1.73	0.75	3.64
80µg/ml	82.11	13.65	2.36	1.88
160µg/ml	60.09	25.59	10.24	4.08
Doxorubicin_25uM	45.44	31.8	18.54	4.22

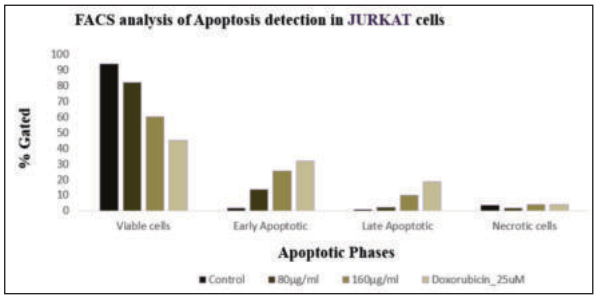

Figure 5: Flow cytometry analysis of Apoptosis detection of JURKAT cells

DISCUSSION:

Dooshivishari agada is a herbo mineral formulation having twelve ingredients in it. In the present study, *Dooshivishari agada* methanolic extract was analysed for inducing apoptosis on the JURKAT cell lines. Many naturally occurring substances exert anticancer effects by induction of apoptotic signaling.[9] Previous studies have revealed that compounds isolated from plants of *Dooshivishari agada* had potent anticancer properties. *Dooshivishari agada* have chemical constituents like Piperin, Piper longumin, Geraniol, Carvone, Angelicin, Patchouli, Ellagic acid, Betulin, Bornneol, Farnesol, Diosgenin, Rutin, Baicalein, Prunetin, Valtrate, citric acid, Costunolide, sesquiterpenes, Glycyrrhizin, Alpha-santalol, palmitic acid, etc. and they are proved for its anticancer activity by previous research works. The majority of these chemical constituents produce effect through a common molecular mechanism that is involved in the acquisition of cancer hallmarks, particularly those that involve the cell cycle and apoptosis across various types of cancer. They specifically suppress the tumor growth, but do not affect the normal physiology at an individual level and also inhibit the angiogenesis.[7]

Dooshivisha (cumulative poison) is mainly having *Kapha pradhanata* (*Kapha* predominancy) and *avarana* (covering property), which gets pacified by *Dooshivishari agada*, as all the ingredients of *Dooshivishari Agada* is having *Kapha-Vatahara* (reduces *kapha* and *vata*) and *tridoshahara* (reduces *kapha*, *pitta*, *vata*) properties.[1]

CONCLUSION:

Methanolic extract of *Dooshivishari agada* was able to induce apoptosis in the cell lines tested. It showed a dosage-dependent increase of apoptotic fragments at the tested doses. Quantitative analysis using the Annexin V/PI assay as an indicator of apoptosis and necrosis demonstrated that methanolic extract of *Dooshivishari agada*, having apoptotic activity, also possesses the minimal capacity of inducing necrotic cell death on JURKAT cells.

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