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Cytotoxic and DNA Fragmentation Activity of *Dooshivishari agada* on Human T-Cell Acute Lymphocytic Leukemia (JURKAT) Cell Lines

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Deepa P[1*], Nataraj H R[2], Anushree C G[3]

[1,3]PG Scholar, [2]Associate professor, Department of Agada tantra, Sri Dharmasthala Manjunatheshwara College of Ayurveda and Hospital, Hassan,India.

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ABSTRACT

Background: *Dooshivisha* (cumulative toxin) is a unique concept of *Agada tantra*, and it is a type of *kritrimavisha* (artificial poison). It is a transformed state of poison when gets cumulatively deposited in the body in day-to-day life. It is the cause of the majority of health issues in the present era. Cancer is one such disease where *Dooshivisha* (cumulative toxin) is the cause. Leukemia is a type of cancer of white blood cells, which has a similar etiology and pathology to *Dooshivisha* (cumulative toxin). *Dooshivishari Agada* is the formulation of choice for the management of *Dooshivisha* (cumulative toxin). **Objectives:** To elucidate the cytotoxicity and DNA fragmentation induced by methanolic and aqueous extract of *Dooshivishari agada* (DVA) on Human T cell acute lymphocytic leukemia (JURKAT) cell lines. **Materials and methods:** JURKAT cells were cultured and treated with methanolic and aqueous extract of DVA (0, 10, 20, 40, 80, 160, 320µg/ml). After 24 hours MTT assay was performed by adding an MTT reagent to assess Cytotoxicity and Doxorubicin was taken as standard. JURKAT cells were cultured and treated with methanolic extract of DVA (80, 160µg/ml). DNA fragmentation was assessed by gel electrophoresis; H_2O_2 was taken as the positive control. **Results:** DVA methanolic extract showed an IC_{50} value of 147.4µg/ml in JURKAT cells. DNA fragmentation was comparatively better at higher concentrations. **Conclusion:** Methanolic extract of DVA possess cytotoxicity and DNA fragmentation activity at higher concentrations.

INTRODUCTION

Dooshivisha (cumulative toxin) is a transformable state of *visha* (poison). These attenuated or denatured poisons function as the latent toxin in the body. The *visha* (poison) either *sthavara* (plant) or *jangama* (animate) has become old and stays inside the body for a long duration (*jeerna*), the potency of which has been reduced (*veeryaheena*) by the effect of *vishaghnaaushadhi* (anti-poison medications), dried by the *davagni* (forest fire), *Vata* (breeze), *atapa* (sunlight) or that which by its very nature is not endowed with all the ten properties of visha (few of the gunas are missing among ten vishagunas), such *visha*(poison) is termed as *dooshivisha*(cumulative toxin). [1-3]In the present era of modernization, people are exposed to different kinds of toxins in their day-to-day life. It gets deposited in the body in small quantities. Throughout getting favorable conditions it produces gravious diseases. Cancer is one such disease that has cumulative toxin as the causative factor. Leukemias are malignant disorders of hematopoietic stem cells, associated with increased numbers of white cells in the bone marrow and/or peripheral blood [4]. It is the 11th most common cancer worldwide[5]. 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[6]. Cancer has a similar etiology and pathology to*dooshivisha*(cumulative toxin). Hence *dooshivisha*(cumulative toxin) can be correlated with Cancer. *Dooshivishariagada* is a herbo mineral formulation, explained chiefly for the management of *dooshivisha*(cumulative toxin). This formulation contains twelve ingredients and the majority of the ingredients are individually proven to have anti-cancer properties through previous research works.[7]

The MTT assay is a means of measuring the activity of living cells via mitochondrial dehydrogenases. The key component is MTT (3-[4, 5- dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) is a water-soluble tetrazolium salt yielding a yellowish solution when prepared in media or salt solutions lacking phenol red. Dissolved MTT is converted to an insoluble purple formazan by cleavage of the tetrazolium ring by mitochondrial dehydrogenase enzymes of viable cells. This water-insoluble formazan can be solubilized using DMSO, acidified isopropanol, or other solvents (Pure propanol or ethanol). The resulting purple solution is spectrophotometrically measured.[8]

DNA fragmentation is a biochemical hallmark of apoptosis. In dying cells, DNA is cleaved by an endonuclease that fragments the chromatin into nucleosomal units, which are multiples of about 180-200 base pairs oligomers and appear as a DNA ladder when run on an agarose

gel. The detection of apoptosis in cultured cells relies heavily on techniques involving the extraction of nuclear DNA and characterization of such oligonucleosomal ladders by gel electrophoresis.[9]

Objectives: To assess the cytotoxic activity of methanolic and aqueous extracts of *Dooshivishari agada* on Human T-cell acute lymphocytic leukemia (JURKAT) cell lines using MTT assay, and to assess cell death by detecting DNA fragments using agarose gel electrophoresis.

MATERIALS AND METHODS:

Table No. 1 Ingredients of *Dooshivishari agada* as per Ashtanga Hrudaya[10]

Sl.No.	*Dravya* (Drug)	Botanical name	Part used
1.	*Pippali*	*Piper longum* Linn.	*Phala* (Fruit)
2.	*Dhyamaka*	*Cymbopogon martini (*Roxb.) W. Watson.	*Panchanga* (Whole plant)
3.	*Jatamansi*	*Nardostachysjatamansi*(D.Don) DC.	*Moola* (Root)
4.	*Lodra*	*Symplocosracemosa*Roxb.	*Twak* (Bark)
5.	*Ela*	*Elettaria cardamomum* (L.) Maton.	*Beeja* (Seed)
6.	*Suvarchika*	*Tribulus terrestris* Linn.	*Phala* (Fruit)
7.	*Kutannata*	*Oroxylum indicum* (L.) Kurz	*Moola* (Root)
8.	*Natha*	*Valerianawallichi* DC.	*Moola* (Root)
9.	*Kushta*	*Saussurealappa*Clarke.	*Moola* (Root)
10.	*Yashtimadhu*	*Glycyrrhiza glabra* Linn.	*Moola* (Root)
11.	*Chandana*	*Santalum album* Linn.	*Kanda sara* (Heart wood)
12.	*Gairika*	Red ochre	-

Materials – MTT assay

1. MTT Powder (the solution is filtered through a 0.2 µ m filter and stored at 2–8 ^{0}C for frequent use or frozen for extended periods)

2. DMSO (Dimethyl sulfoxide)

3. CO_2 incubator

4. Spectramax I3X Plate reader

5. DMEM (Dulbecco's Modified Eagle medium) plain media

6. Fetal Bovine Serum (FBS), penicillin (100 IU/ml), streptomycin (100 µg/ml) for cell culture

Materials – DNA fragmentation

1. Lysis buffer

2. TE buffer

3. Trypsin

4. RNase A

5. 3M Sodium Acetate

6. 100% Ethanol

Preparation of the formulation:

- All authenticated twelve ingredients were checked for any impurities and cleaned.

- *Gairika* is one of the ingredients of DVA which is a mineral ingredient. Hence shodhana should be done before use.

- 10gm of *gairika* was taken and powdered. Cow's ghee was taken in a frying pan, to this powdered *gairika* was added and fried on a medium flame till the color changed to slightly brown.[11]

- The remaining eleven ingredients were made into coarse powder by using *khalva yantra*(pestle and mortar) separately.

- 10gm of each of the coarse powders of 12 ingredients are then mixed to form a homogeneous mixture.

A. Cytotoxicity studies for JURKAT cell line: MTT assay

Preparation of test solutions

For cytotoxicity studies, 32mg/ml were prepared using DMSO and serial two-fold dilutions were prepared from 320µg/ml to 10µg/ml using DMEM (Dulbecco's Modified Eagle Medium) plain media for treatment.

Cell lines and culture medium

JURKAT cells were procured from ATCC (American Type Culture Collection), and stock cells were cultured in RPMI (Roswell Park Memorial Institute medium) supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/ml), streptomycin (100 µg/ml) in a humidified atmosphere of 5% CO2 at 37oC until confluent. The cell was dissociated with cell dissociating solution (0.2% trypsin, 0.02% EDTA (Ethylene diamine tetraacetic acid), 0.05 % glucose in PBS (Phosphate-buffered saline)). The viability of the cells is checked and centrifuged. Further, 50,000 cells/well were seeded in a 96-well plate and incubated for 24hrs at 37^{o}C, under a 5% CO_2incubator.

Procedure

The suspension cell culture was centrifuged and the cell count was adjusted to 1.0 x 105 cells/ml using respective media containing 10% FBS. To each well of the 96-well microtiter plate, 100 µl of the diluted cell suspension (50,000 cells/well) was added and incubated for 24hrs at 37oC, a 5 % CO2 incubator. After 24 h, 100 µl of different test concentrations (10, 20, 40, 80, 160, 320µg/ml) of aqueous and methanolic extracts of DVA were added to the suspension in microtiter plates. 100 µl of Standard Doxorubicin at different concentrations (0, 3.125, 6.25, 12.5, 25, 50,100 µM) were added to the suspension and the media serves as the negative control. The plates were then incubated at 37oC for 24hrs in a 5% CO2 atmosphere. After incubation 10µl of MTT (5 mg/ml of MTT in PBS) was added to each well. The plates were incubated for 4h at 37o C in a 5% CO2 atmosphere. Then the supernatant was collected, centrifuged, and to the pellet 100 µl of DMSO was added. This solution was then transferred to their respective wells and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 590 nm. The percentage growth inhibition was calculated using the following formula and the concentration of test drug needed to inhibit cell growth by 50% (IC50) values is generated from the dose-response curves for each cell line.

B. DNA fragmentation studies on the JURKAT cell line:

Cells were seeded at a concentration of 10^6 per 35mm dish and incubated at 37ºC / 5% CO_2 for 24 h. The confluent cells grown after 24 hours of incubation were treated with DVA at 80µg/ml and 160µg/ml, and with H_2O_2 treatment at 200µM as standard. After treatment, cells were trypsinized, and both adherent and floating cells were collected by centrifugation at 2000 rpm for 5 min. The cell pellet was suspended in 0.5 ml lysis buffer (pH 7.8) [Tris-HCl 10 mM, pH 8; EDTA 20 mM, pH 8.0; TritonX-100 0.2% or Sodium-*N*-lauroyl sarcosinate, 4M NaCl], vortex vigorously and incubated at 50°C for 5 min. To the lysate, 0.5 ml of phenol-chloroform isoamyl alcohol was added and mixed for 2-3 minutes. In this stage protein, part, and DNA parts of the cell get separated by dissolving in phenol and water portion of chloroform respectively. It was centrifuged at 10000 rpm for 15 min at 4ºC. The water portion of chloroform with DNA stays in the upper portion and phenol with proteins settles at the bottom after centrifugation. The upper aqueous layer was taken in a new tube, to which double the volume of cold 100% ethanol was added and 3M sodium acetate was added (The final concentration of sodium acetate was 0.3 M). It was incubated for 5-10 minutes at room temperature. Centrifuged at 10000 rpm for 15 min and discard the supernatant. After removing the supernatant, the DNA pellet was washed in 70% ethanol and centrifuged at 5000 rpm for 10 minutes. The supernatant was removed, the DNA pellet was air-dried and was finally dissolved in TE buffer (Tris-HCl 10 mM, pH 7.4, EDTA 1 mM, pH 8.0), and separated by 2% agarose gel electrophoresis at 100 V for 50 min.

Observation and Result

A. MTT assay for JURKAT cell line

MTT assay determines the percentage of cell viability by producing a purple stain. If more of the purple color is observed by an inverted light microscope, more will be cell viability and less percentage inhibition. This is expressed as OD value. In both samples there was increased OD with the increase in concentration, hence there is a decrease in cell viability with the increase in concentration.

Table No. 2 – MTT assay for JURKAT cell line using Doxorubicin

JURKAT				
Test compounds	**Conc. µM**	**OD @ 590nm**	**% Inhibition**	**IC50 Value in µM**
Doxorubicin	0	0.564	0.00	**18.51**
	3.125	0.476	15.72	
	6.25	0.410	27.26	
	12.5	0.367	34.88	
	25	0.249	55.95	
	50	0.205	63.71	
	100	0.135	75.99	

Standard doxorubicin showed more than 50% inhibition at the concentration of 25 µM, 50 µM, and 100 µM, and the % inhibition was found to be 55.95, 63.71, and 75.99 respectively.

Table No. 3 – MTT assay for JURKAT cell line using DVA methanol extract

JURKAT				
Test compounds	**Conc.µg/ml**	**OD @ 590nm**	**% Inhibition**	**IC50 Value in µg/ml**
DA Methanol Extract	0	0.564	0.00	**147.4**
	10	0.503	10.94	
	20	0.454	19.60	
	40	0.418	25.84	
	80	0.374	33.74	
	160	0.268	52.58	
	320	0.205	63.76	

JURKAT cells treated with 0, 10, 20, 40, 80, 160, 320 µg/ml concentrations of methanolic extract of *Dooshivishari agada*, showed the percentage inhibition of 0, 10.94, 19.60, 25.84, 33.74, 52.58, and 63.76 respectively.

When the JURKAT cells are treated with 0, 10, 20, 40, 80, 160, and 320µg/ml concentrations of aqueous extract of *Dooshivishari agada*, showed the % inhibition of7.64, 11.15, 20.65, 26.14, 34.63 and 38.30 respectively after 24 hours of treatment.

Table No. 4 – MTT assay for JURKAT cell line using DVA aqueous extract

Test compounds	**Conc.µg/ml**	**OD @ 590nm**	**% Inhibition**	
DA Aqueous Extract	10	0.521	7.64	**IC_{50} Value was not calculated due to lesser % inhibition**
	20	0.501	11.15	
	40	0.448	20.65	
	80	0.417	26.14	
	160	0.369	34.63	
	320	0.348	38.30	

Methanolic extract of *Dooshivishari agada* was found to be highly significant than the aqueous extract of DVA in all intervals.

At 160 µg/ml and 320 µg/ml concentrations of methanolic extract of DVA, more than 50% of cell death is observed.

The cytotoxic activity of the sample can be determined by comparing the IC_{50} value of the sample with a standard. The IC_{50} values for the cytotoxicity tests were derived from nonlinear regression analysis (curve fit) based on the sigmoid dose-response curve (variable).

Dooshivishari agada methanolic extract showed an IC_{50} value of 147.4 µg/ml inhibition in JURKAT cells. Standard doxorubicin showed an IC_{50} value of 18.51 µM inhibition in the same. Aqueous extract of DVA did not show significant activity, hence IC_{50} was not calculated.

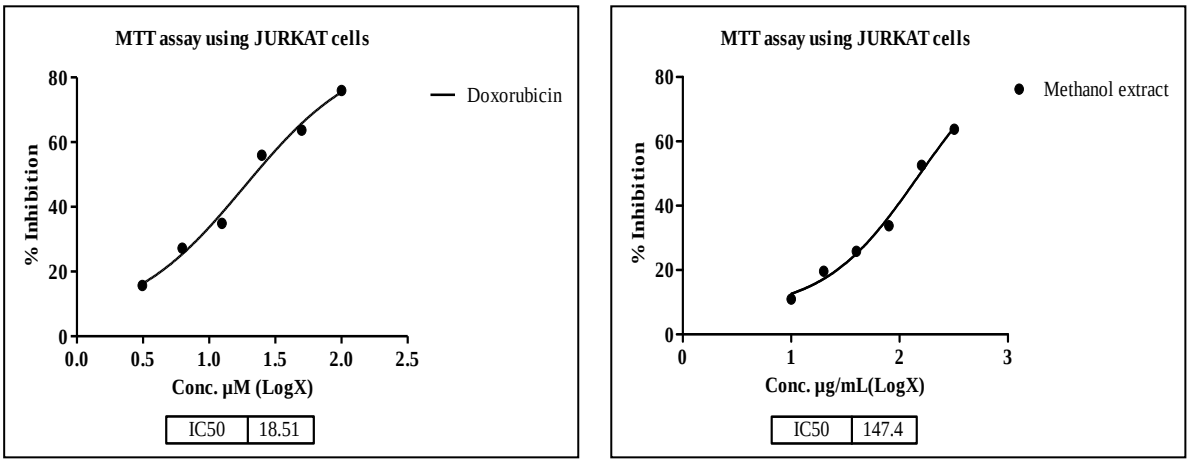


Fig. No. 1: The significant activity of the (A) standard doxorubicin and (B) DVA methanol extracts against JURKAT viable cells in the culture medium

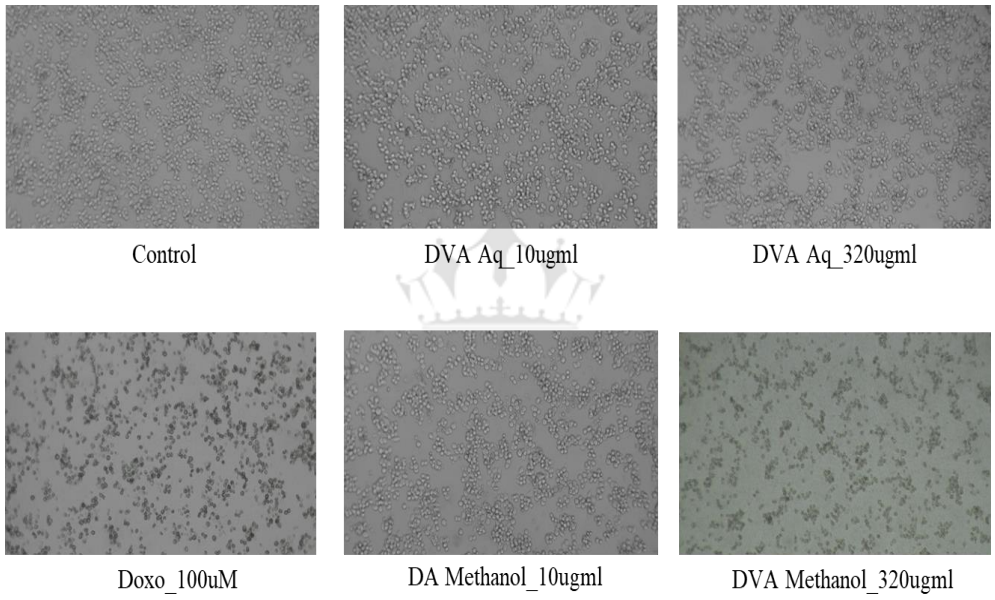
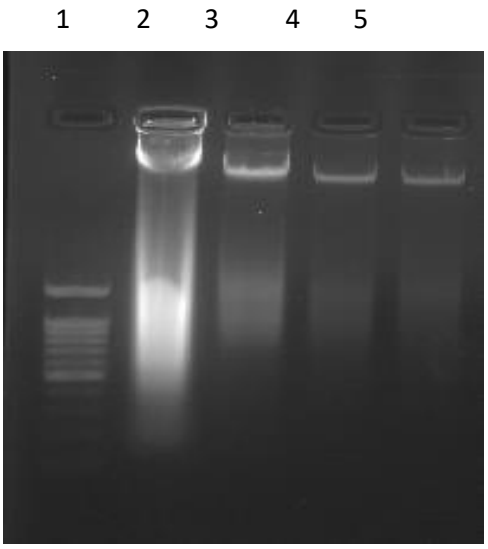


Figure. No. 2: Morphologic changes of cells after treatment with different concentrations of aqueous and methanolic extracts of DVA

After 24 hour co-culture of the cells with different concentrations of aqueous and methanolic extracts of *Dooshivishari agada* (0, 10, 20, 40, 80, 160, 320 µg/ml), the cell population was decreased as compared to the control and also morphological changes were observed in the JURKAT cells, which consisted of reduction in the number of living cells, cell volume and rounding until the nucleus constituted the majority of the cellular volume. There was an observationally severe reduction of malignant cells (JURKAT). This cytotoxicity was increased at higher concentrations.

B. DNA Fragmentation analysis



Lane 1 –Ladder,

Lane 2- H_2O_2-200µM,

Lane 3 – *Dooshivishari agada* - 160µg/ml

Lane 4- *Dooshivishari agada*- 80µg/ml

Lane 5 – Control

Figure. No. 3: Gel electrophoresis result of DNA fragmentation

When sample DVA was treated at 80µg/ml and 160µg/ml against JURKAT cells, it was found that DNA fragmentation was comparatively better at higher concentrations. Positive control H_2O_2 treatment at 200µM showed significant DNA damage when compared to the sample.

DISCUSSION

Dooshivisha (Cumulative toxin) is a latent toxin due to the cumulative deposition of a small amount of toxin over a period in the body. It is having mild potency, is not fatal for an individual, and has delayed action. Similarly, cancer also has the cumulative etiology of deposition of toxins in the body.

Cragg and Newman (2000) state in a clinical trial over 50% of the drugs are isolated from natural sources.[12] currently, in chemotherapy, several drugs used are isolated from plant species.[13]The main aim of our study was to determine the effect of DVA on JURKAT cells. DVA is a herbomineral formulation where all its ingredients are proven to have anticancer properties.[7] DVA methanolic extract was also found to have apoptotic activity[14] and induce cell cycle arrest[15] in JURKAT cells through previous studies.

The present study is to examine the effect of aqueous and methanolic extract of *Dooshivishari agada* on cytotoxic activity against Human T-cell acute lymphocytic leukemia

(JURKAT) cells. We found that methanolic extract of DVA showed good cytotoxicity against JURKAT cells, but the aqueous extract of DVA was not effective, based on the IC_{50} value. The high sensitivity of malignant cells may in part reflect the differences in growth rates at different concentrations of test samples. Methanolic extract of DVA inhibited JURKAT cell growth in a dose-dependent manner at 24 hours of treatment. Based on morphologic changes identified by inverse microscopy, morphologic characters of apoptosis and the reduction of the cells were observed [Figure 2].

DNA fragmentation is the biological hallmark of the apoptotic process, which shows the presence of typical ladder DNA fragments of 180-200 base pairs. DNA gel electrophoresis method was used to determine the possible mode of death caused by methanolic extract of DVA. DNA fragmentation was seen in treated cells at different concentrations of methanolic extract of DVA and was comparatively better at higher concentrations. No ladder formation was seen in untreated cells.

CONCLUSION

Methanolic extract of *Dooshivishari agada* showed an IC_{50} value of 147.4µg/ml in JURKAT cells. Hence it can be understood that *Dooshivishari agada* possesses cytotoxic activity on JURKAT cell lines. This was significantly demonstrated through the morphological changes. When sample *Dooshivishari agada* was treated at 80µg/ml and 160µg/ml against JURKAT cells, it was found that DNA fragmentation was comparatively better at higher concentrations.

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