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Research Article

CYTOTOXIC EFFECT OF JUGLONE ON HUMAN PERIPHERAL BLOOD LYMPHOCYTES

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ABSTRACT

Juglone, a naturally occurring naphthaquinone is reported to have several medicinal properties. However, juglone is also reported to be toxic to some cell types. Human peripheral blood lymphocytes were treated with various concentrations of juglone for various time points to assess the toxic effects on the cells. Toxicity studies were performed by the Trypan Blue dye exclusion method and the MTT assay. Decrease in viability percentage was noticed with increasing concentration of juglone in the cell culture system. Cell morphology was then studied using Giemsa staining and fluorescence microscopy and a significant increase in the apoptotic features were noticed in the juglone treated cells. SDS-PAGE, and silver staining and Western blotting were performed to study the protein expression. DNA fragmentation was assessed by TUNEL assay. The apoptotic cells were then studied by morphological evaluation. It was observed that exposure to juglone (5-50 µM) resulted in a concentration and time dependent decrease in cell viability. From TUNEL assay, fragmentation of DNA was observed there by apoptotic cell death was confirmed.

Keywords: Quinones, Apoptosis, cell membrane blebbing, Juglone.

INTRODUCTION

Quinones comprise a large group of naturally occurring and synthetic compounds that have functional, toxicological, mutagenic, and antitumor actions. Quinones are widespread in nature [1] and have a variety of functions in the life cycles of most kinds of living organisms. Both plants and insects use quinones as defensive compounds, examples of which are juglone in the walnut tree and hydroquinone in bombardier beetles [2]. Juglone (5-hydroxy-1,4-naphthoquinone) is a naturally occurring naphthoquinone in the leaves and green shell of the fruits of *Juglacea,* e.g. walnut trees. But juglone prevents the growing of plants and has also been tested in human pharmacology as a cytotoxic agent. Apoptosis is observed in all metazoans include the both plant and animals. Apoptosis, also called programmed cell death,in which the cell dies by its own action [3] and plays a critical role in the development of multicellular organisms. Apoptosis is a highly conserved, precisely regulated cascade of biochemical and molecular events for eliminating aged and sub-lethally damaged cells, and can be induced by various chemical and mechanical damages. Morphological changes in the apoptotic cells include membrane blebbing, loss of membrane asymmetry and attachment, cell shrinkage, chromatin condensation, and chromosomal DNA fragmentation [4]. Although studies have indicated that there is extensive two-way translocation of proteins between organelles and the cytosol. Release of cytochrome c from the mitochondria leads to what may be the ultimate mechanisms of apoptosis, namely, formation of the apoptosome, activation of procaspase-9, and initiation of the caspase cascade.

The lymphocytes are the central cell of the immune system responsible for adaptive immunity and immunological attributes of diversity specificity, memory and self/non self recognition. Lymphocytes in the human body the lymphocytes continually circulate in blood and lymph and are capable of migrating into tissue spaces and lymphoid organ, thereby integrating the immune system to the high degree [5].

In the immune system, apoptosis is required for lymphocyte development and homeostasis. The dysregulation of apoptosis leads to a variety of immune disorders. Too little cell death of activated lymphocytes can result in autoimmune disorders and too much cell death can lead to immunodeficiency. In our laboratory, the three anthraquinones rhein, dantron, and chrysophanol were compared regarding their toxicity and their ability to induce apoptosis in primary rat hepatocytes, and only rhein was found to have redox-cycling capacity and induce apoptosis. These results are in accordance with previous observations, in that they show that the toxicity of the quinone is substantially altered by the chemical properties of the substituents. Since DPPD and the iron chelator desferal completely inhibited apoptosis, we concluded that the cytotoxic effect of rhein was due to formation of free radicals and lipid peroxidation. In another study (Roberg and naphthazafin-treated rat heart myocytes underwent apoptotis but no lipid eroxidation was detected; the apoptotic death of those cells was, however, inhibited by preincubation with the lipid-soluble antioxidant alpha-tochopherol succinate, which is an effective free radical scavenger. Studies of the oxidation products of catecholamines (i.e., seretonin, dopamine, dopa, adrenaline, and noradrenaline) have indicated that protein oxidation by quinones may lead to apoptosis. Oxidation results in formation of *ortho*-quinones, which contribute to cytotoxicity and have been suggested to underlie disorders such as rkinson's disease and schizophrenia. In soluble brain extracts, *o*-quinones have been bserved to undergo redox cycling and covalent binding to the free sulfhydryl groups of proteins. Experiments *in vitro* have shown that this binding is enhanced by Fe (II), which results in the formation of high-molecular protein aggregates, lipid peroxidation. The investigators have proposed that these effects can be prevented by conjugation of *o*-quinones to glutathione and that they are involved in protecting the cells of the nervous system from degenerative processes [2].

MATERIALS AND METHODS

Isolation of human peripheral blood lymphocytes

The isolation of lymphocytes from peripheral blood collected from healthy human volunteers was carried out according to the method of Boyum A., 1968 [6], which employs density gradient centrifugation of blood cells using Ficoll-Hypaque (HISTOPAQUE 1077). This is a solution containing polysucrose and sodium diatrizoate adjusted to a density of 1.077 ± 0.001 g/ml that facilitates rapid recovery of viable lymphocytes from whole blood. The lymphocytes in dispersion were checked for cell viability by trypan blue dye exclusion method. 10µl of the cell suspension was mixed with 2µl of trypan blue and was loaded onto a hemocytometer for counting (the four large squares were used for counting). Viable cells are unstained while non-viable cells are stained blue.

$$\text{Cells/ml} = \frac{\text{Average number of cells per large square} \times 10^4}{\text{Dilution factor}}$$

Assessment of toxicity of juglone on lymphocyte cell culture

In order to investigate whether juglone is cytotoxic to lymphocytes, the cells were cultured in the presence of different concentrations of juglone ranging from 2 to 50 µM for varying periods of time, their viabilities were assessed and comparisons were made with untreated cells. The cells were also treated with dimethyl sulphoxide alone to check for any variations of the control from the dimethyl sulphoxide control. Viability was measured by trypan blue dye exclusion method and MTT assay.

Trypan blue dye exclusion method

Trypan blue dye exclusion is a cell viability assay based on the ability of the live cells to exclude the vital dye, trypan blue Talwar G.P. 1983[7]. The dye penetrates the membrane of non-viable cells as the cells lose the integrity of the cell membrane, hence are stained blue, and can therefore be distinguished from viable cells. The viable cells appear round and glossy while the non-viable cells appear bloated in size and are blue in colour.

$$\text{Percentage viability} = \frac{\text{Average number of cells per large square (treated) Average}}{\text{number of cells per large square (untreated)}} \text{ x100}$$

MTT assay

MTT assay is a non radioactive colorimetric procedure, which is used to measure the cell viability in response to a variety of cytotoxic stimuli. The assay is based on the reduction of the yellow, water soluble tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) within metabolically active cells Mosmann T., 1983 [8] Camping BG, 1991 [9]. The reduction of the tetrazolium salt occurs by the action of mitochondrial dehydrogenases which present only in viable cells, yields a purple formazan product which can be quantified spectrophotometrically. The percentage viability of the treated cells was calculated by comparing it with the control cells which were not treated with juglone.

$$\text{Percentage viability} = \frac{\text{Net absorbance of treated cells}}{\text{Net absorbance of untreated cells}} \text{ x100}$$

Assessment of cell morphology in juglone treated cells

Peripheral blood lymphocytes were cultured in varying concentrations of juglone for different time periods and their morphology was studied using Giemsa staining and fluorescence microscopy.

Giemsa Staining

Giemsa stain is a solution containing a mixture of dyes like methylene blue related azures (basic dyes) and eosin (acidic dye). The basic dyes carry net positive charges, consequently they stain the nuclei of white blood cells blue. Hence the morphology of cells treated with juglone can be studied by staining them with Giemsa thereby enabling the observation of defined cellular features that characterize cell death due to apoptosis. The morphology of the cells is examined under 100x objective of the microscope (Nikon Eclipse E600 POL).

Fluorescence Microscopy

Cell death can be studied morphologically by using differential fluorescent dyes like acridine orange and ethidium bromide. Acridine orange-Ethidium bromide staining uses a combination of two dyes to visualize cells with aberrant juglone organization. Acridine orange was used to visualize the number of cells which has undergone apoptosis, but it cannot distinguish viable from non-viable cells. To achieve this, a mixture of acridine orange and ethidium bromide was used. Acridine orange can penetrate viable cells and stain them green while ethidium bromide, which is excluded by viable cells, stains the nonviable cells, orange. This is because ethidium bromide can enter the cells only if the cell membrane is ruptured. The intact cell membrane is impermeable to ethidium bromide. The differential uptake of these two dyes allows the identification of viable and non-viable cells. The excitation and emission wavelengths for acridine orange are 502 and 526 nm while for ethidium bromide they are 510 and 595 nm. The fluorescence emitted and the morphology of the cells was observed in a fluorescence microscope (Nikon Eclipse E600 POL) using an appropriate filter.

Isolation and Separation of cellular proteins

The peripheral blood lymphocytes were cultured in varying concentrations of juglone for 3, 6, 12, 24, 48 and 72 hours. The cells were lysed in a buffer containing 50mM HEPES, 150mM Sodium chloride, 1% triton X-100, 2mM Sodium orthovanadate, 1mM PMSF, 10µg/ml leupeptin and 10µg/ml of aprotinin for 30 minutes in ice. The whole cell lysates were centrifuged at 4°C for 13000 rpm for 20 minutes. The total protein concentration was determined using Bradford assay and the results were used to calculate the amount of each of the samples to be loaded to be analysed using SDS- PAGE.

Bradford assay

Protein concentration was estimated by the Bradford CBB G-250 dye binding assay, using bovine serum albumin as standard. The assay is based on the principle that when proteins bind to the Coomassie Brilliant Blue G-250 (CBB G-250) dye a shift in the absorbance maximum from 465 to 595 nm occurs. The concentration of protein in the cell lysate was estimated from the standard graph.

Sodium Dodecyl Sulphate Polyacylamide Gel Electrophoresis (SDS-PAGE) of proteins:

SDS-PAGE was carried out according to the method of Laemmli U.K., 1970 [10] using a 10% separating gel. The samples were loaded in each well using a Hamilton's syringe and electrophoresis was carried out at 100 V till the dye front reached the bottom of the gel. The gel was taken for western blotting.

Silver Staining-Silver staining fast procedure was followed to clearly visualize the bands formed on running the SDS PAGE gel.

Western Blotting

The method originated from the laboratory of George Stark at Stanford. The name "Western blot" was given to the technique by W. Neal Burnette [11]. The western blotting technique was carried out using a standardized protocol. The nitrocellulose membrane was then taken for detection wherein equal volumes of both the reagents were mixed and added to the membrane on the side containing the blot. It was incubated for 90 seconds after which the excess detection reagent was drained off. The membrane was wrapped in a fresh piece of polythene sheet and placed in an X-ray film cassette with the blot side facing up. A sheet of autoradigraphy film was placed on top of the nitrocellulose membrane. The cassette was closed and exposed for 2 minutes. After two minutes the film was removed and a fresh film was placed for further exposure. The film was developed using Kodak developer solution and fixed with sodium thiosulphate.

DNA fragmentation detection studies

Tunel assay

Tunel assay is a non-isotropic system for the labeling of DNA breaks in apoptotic nuclei in paraffin-embedded tissues sections, tissue cryosections and in cell preparations fixed slides. Detection of apoptotic cells is done using a molecular biology-based end-labelling, histochemical or cytochemical technique. Fragmentation End Labelling(FragEL) of DNA is done using a standard kit bought from Calbiochem, California, USA.

Terminal deoxynucleotidyl transferase (TdT) binds to exposed 3'-OH ends of DNA fragments generated in response to apoptotic signals and catalyses the addition of biotin labeled and unlabelled deoxynucleotides. Diaminobenzidine(DAB) reacts with the labeled sample to generate an insoluble coloured substrate at the site of DNA fragmentation. Counter staining with methyl green aids in

morphological evaluation and characterization of normal and apoptotic cells viewed under a light microscope (Nikon Eclipse E600 POL).

RESULTS

Assessment of cytotoxicity of juglone on lymphocyte cell culture

The cytotoxic effect of juglone of different concentrations for various periods of time was assessed from the percentage viability of the lymphocytes by the trypan blue dye exclusion method and also by performing the MTT assay.

Trypan Blue Dye Exclusion Method

The viability of lymphocytes under the influence of juglone of varying concentrations was assessed by trypan blue dye exclusion method and was then compared with the control cells. The viability of cells treated with juglone was found to decrease, as shown by the results obtained from trypan blue dye exclusion method. Concentrations of juglone of 5µM and less were not found to affect the viability of the cells by any significant amount. Cell viability was found to decrease in cells treated with higher concentrations of juglone (10-50 µM). A decrease in number of viable lymphocytes with increase in concentration of juglone in the range of 10-50µM was observed. At concentrations of under 5µM cell viability of over 95% was observed in the first 12 hours of incubation. Higher concentrations like 10µM- 50µM found a drop in viability of up to 65% in 24 hours. With increasing time of incubation, the viability of the juglone-treated lymphocytes underwent a significant decrease. Concentrations of 10µM and above were found to be toxic to the lymphocytes. The viability was found to be as low as 20% at a concentration 50µM, after an incubation of 72 hours. Percentage of viable cells after the treatment of juglone at various concentrations and time points are tabulated **(Table-1).**

Table 1: Percentage Viability of Lymphocytes Treated with Juglone Assessed by Trypan Blue Dye Exclusion Method

Concentration (µM)	Percentage Viability (%)			
	12h	24h	48h	72h
DMSO Control	100	100	100	100
J2	96	94	87	74
J5	97	87	70	65
J10	93	85	55	40
J20	89	80	51	36
J50		67	31	20

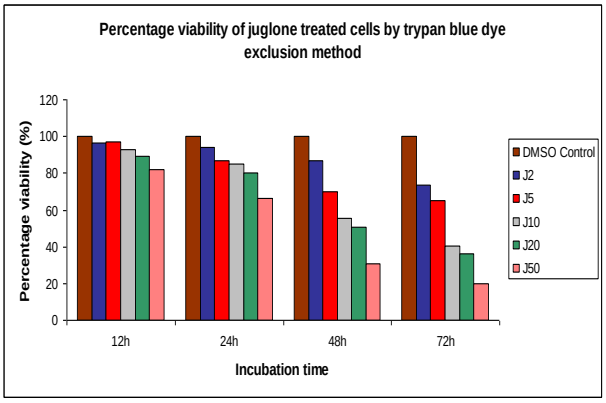


Figure 1: Percentage Viability of Lymphocytes Treated with Juglone Assessed by Trypan Blue Dye Exclusion Method

MTT Assay

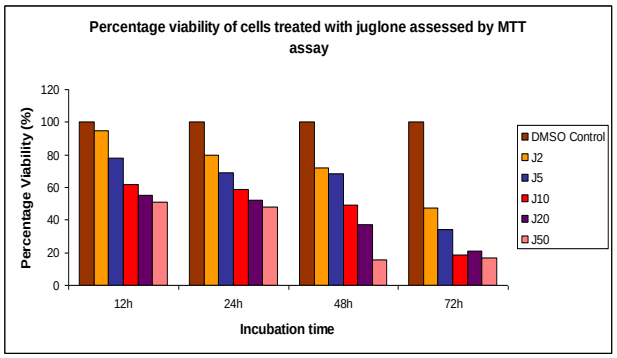
The metabolic activity and hence the viability of the lymphocytes was evaluated through the MTT assay, after treatment of the lymphocytes with varying concentrations of juglone at several time points, including 12, 24, 48 and 72 hours. The percentage viability of the lymphocytes treated with 10µM or more of juglone found a significant decrease, indicating the toxicity of juglone to lymphocytes as shown in **table 2** and **graph**.

In this study, the toxicity of juglone to human peripheral blood lymphocytes was assessed and the results obtained were similar to those got in the trypan blue dye exclusion method. The viability of cells treated with juglone was found to decrease, as shown by the results obtained from trypan blue dye exclusion method. Concentrations of juglone less than 10µM were not found to affect the viability of the cells by significant amounts. Cell viability was found to decrease in cells treated with higher concentrations of juglone (10-50µM). The viable cell percentage dropped drastically at a concentration of 50µM, on incubation. After 72 hours of juglone treatment, the viability of the lymphocytes was found to be less than 20% at concentrations above 10µM.

Table 2: Percentage of viable cells at various time points of the control and the juglone treated cells assessed using MTT assay

Concentration (µM)	Percentage Viability (%)			
	12h	24h	48h	72h
DMSO Control	100	100	100	100
J2	95	80	72	48
J5	78	69	68	34
J10	62	59	49	19
J20	55	52	39	21
J50	50	48	15	17

Figure 2: Percentage of viable cells at various time points of the control and the juglone treated cells assessed using MTT assay



Assessment of cell morphology in juglone treated cells

Human peripheral blood lymphocytes were cultures in varying concentrations of juglone for different time periods and their morphology was studied using Giemsa staining and fluorescence microscopy.

Giemsa Staining

Giemsa stain is a DNA staining solution which stains the nuclei of lymphocytes blue, thereby enabling the study of nuclear morphology and the changes associated with it when cells undergo different cell death processes. The nuclei stain blue in colour while the cytoplasm stains pink. Cell death due to apoptosis is characterized by certain cellular morphological features like cell membrane blebbing, changes to the cell membrane such as loss of membrane asymmetry and attachment, cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation. The morphology of the cells showed significant alterations with increasing concentrations of juglone and also with increasing incubation periods. The appearance of condensed chromatin and fragmented nuclei (see **Figures-6-11**) indicated that juglone mediates cell death through apoptosis. The apoptotic, necrotic and viable cells were counted and tabulated (see **Tables-3-5** and **Graphs**).

Even after 48 hours of incubation, concentrations less than 10µM were not found to be toxic to the cells. However, concentrations of 10µM and above were found to be toxic after 24 hours of incubation;

with a decrease in viability to 68% at a concentration of 50µM. These cells exhibited chromatin condensation, formation of apoptotic bodies, nuclear fragmentation and membrane blebbing, indicating that juglone induces apoptosis in lymphocytes. Necrosis was another feature which was observed in the cells, especially at higher concentrations (up to 43% of the total cell population upon incubation for 72 hours); further strengthening the point that juglone is toxic to lymphocytes.

Table 3: Percentage of apoptotic, necrotic and viable cells, at 24 hours in juglone treated cells, determined using Giemsa staining

Concentration (µM)	Percentage (%)		
	Apoptotic	Necrotic	Viable
DMSO Control	2	1	97
J1	3	0	97
J2	5	1	94
J5	9	3	88
J10	10	4	86
J20	20	8	72
J50	22	10	68

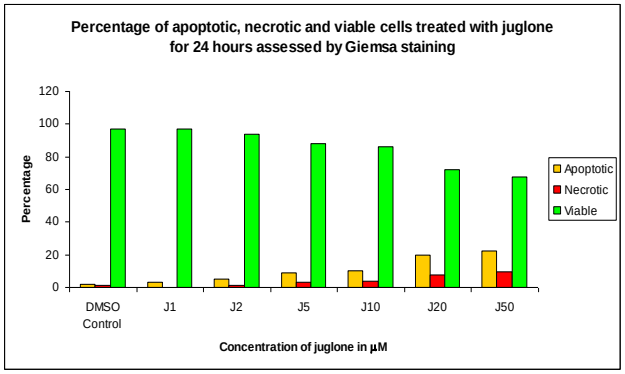


Figure 3: Graphical representation of lymphocytes treated with varying concentrations of juglone, incubated for 24 hours and stained using Giemsa stain

Table 4: Percentage of apoptotic, necrotic and viable cells, at 48 hours in juglone treated cells, determined using Giemsa staining

Concentration (µM)	Percentage (%)		
	Apoptotic	Necrotic	Viable
DMSO Control	0	1	99
J1	8	2	90
J2	10	4	88
J5	11	8	81
J10	14	26	60
J20	18	31	51
J50	25	30	45

Table 5: Percentage of apoptotic, necrotic and viable cells, at 72 hours in juglone treated cells, determined using Giemsa staining

Concentration (µM)	Percentage (%)		
	Apoptotic	Necrotic	Viable
DMSO Control	4	1	95
J1	8	7	85
J2	12	14	74
J5	16	26	58
J10	19	34	37
J20	17	39	44
J50	20	43	37

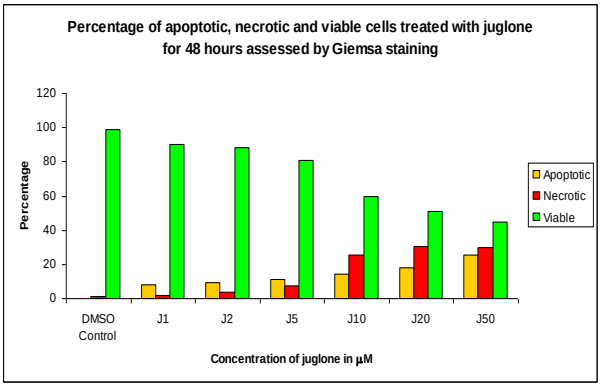


Figure 4: Graphical representation of juglone treated lymphocytes incubated for 48 hours and stained using Giemsa stain

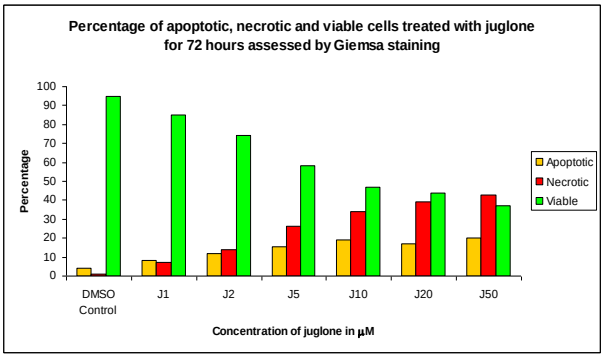


Figure 5: Graphical representation of lymphocytes treated with varying concentrations of juglone, incubated for 72 hours and stained using Giemsa stain

Morphological Analysis of Lymphocytes by Giemsa Staining

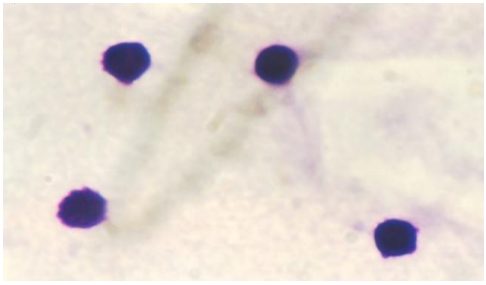


Figure 6: Control cells stained with Giemsa stain after 24 hours of incubation. Note the clear, round cells with complete nuclei indicating normal characteristics

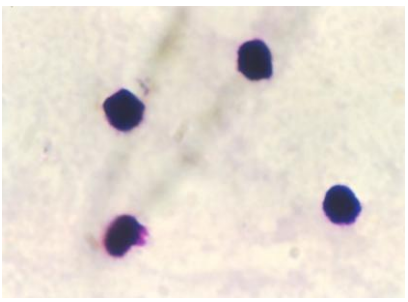


Figure 7: Control cells stained with Giemsa stain after 48 hours of incubation. Note that the cells exhibit normal characteristics

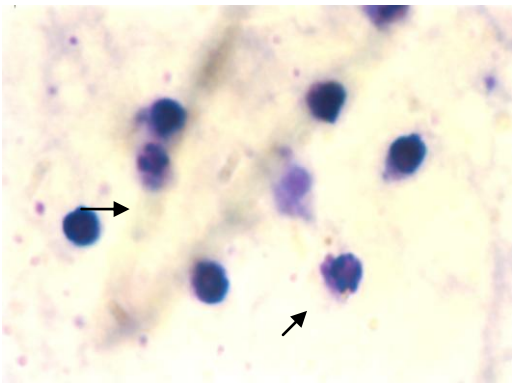


Figure 8:Cells treated with 20µM juglone and stained with Giemsa after an incubation of 24 hours. Note the distinct apoptotic features in some cells (indicated by arrows)

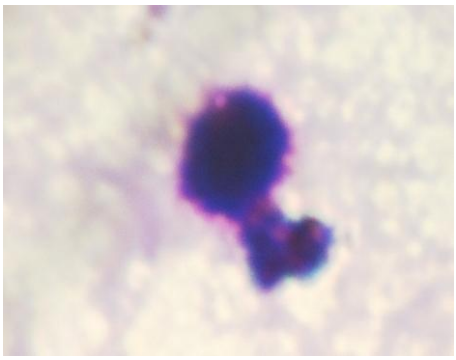


Figure 9: Necrotic cells seen on treatment of lymphocytes with 50µM juglone for 72 hours

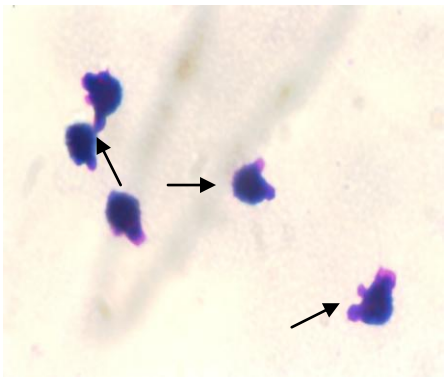


Figure 10: Several cells showing formation of blebs in cells treated with 50µM juglone for 48 hours.

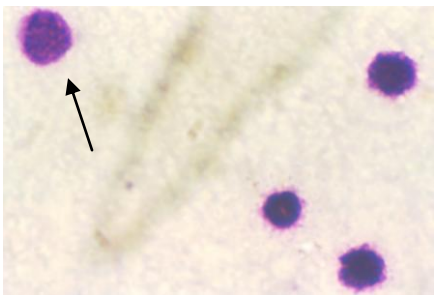


Figure 11: A cell indicate cell with sparsely chromatin, a feature of apoptosis

Fluorescence microscopy

Similar results were observed from fluorescence microscopy studies, in which differential staining using ethidium bromide and acridine orange was done. Acridine orange was used to visualize the number of cells which has undergone apoptosis, but it cannot distinguish the viable from the non-viable cells. To achieve this, a mixture of acridine orange and ethidium bromide was used. Acridine orange can penetrate viable cells and stain them green while ethidium bromide, which is excluded by viable cells, stains the nonviable cells, orange. This is because ethidium bromide can enter the cells only if the cell membrane is ruptured (the intact cell membrane is impermeable to ethidium bromide). The differential uptake of these two dyes allows the identification of viable and non-viable cells.The viability of juglone treated lymphocytes was found to be compromised and characteristic features of apoptosis were noticed, as shown in **Figures (13-15)**, while the control cells gave green fluorescence and had intact cellular morphology as illustrated in **Figure 12.**

Early/viable apoptotic cells exhibit a bright green nucleus showing condensation of chromatin as dense green areas, while the late apoptotic cells have an orange nucleus showing condensation of chromatin, and necrotic cells display an orange nucleus with intact structure. Control cells fluoresced green and had normal cell morphology with intact cell membrane and nucleus, while cells treated with concentrations of juglone greater than 10µM showed many characteristic features of apoptosis including condensation of chromatin, fragmentation of nucleus and membrane blebbing. Viable, non-viable apoptotic and necrotic cells were all observed in lymphocytes treated with concentrations greater than 10µM of juglone.

Upon incubating the cells with 10µM juglone for 24 hours, the percentage of viable cells was as low as 59%, with the apoptotic green cells and the red cells at just over 20% each. When the juglone-treated cells were incubated for 72 hours, the percentage of the viable cells underwent a steep decline, while the apoptotic and necrotic cells' percentage increased greatly, especially that of the necrotic cells (up to 60% necrotic, in cells treated with 50-100µM of juglone).

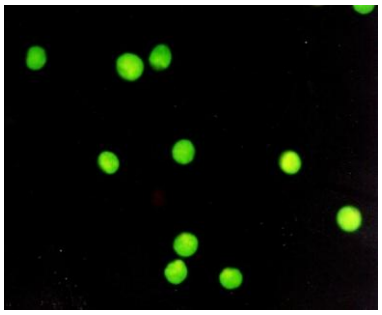


Figure 12: Morphological Analysis of Lymphocytes by Ethidium Bromide

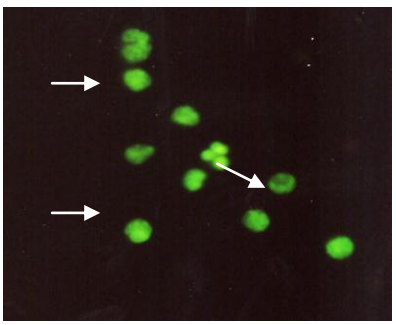


Figure 13: Control cells fluorescing green due to acridine orange uptake

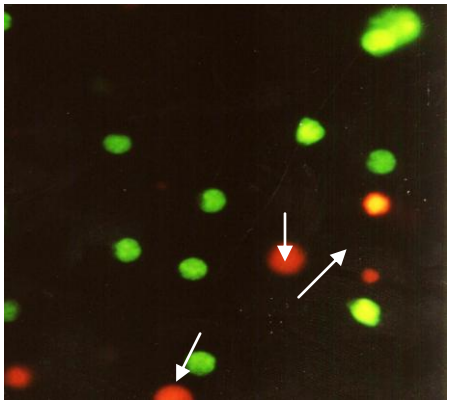


Figure 14: Cells treated with 10µM juglone, stained after 24 hours of incubation (arrows indicate apoptotic cells)

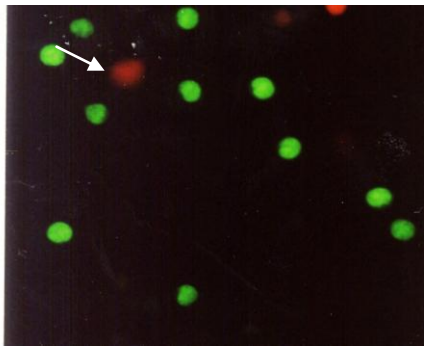


Figure 15: Cells showing the various morphological features seen on ethidium bromide-acridine orange staining of cells treated with 20µM juglone for 48 hours. (Arrows indicate necrotic cells)

Table 6: Ethidium Bromide-Acridine Orange dual staining results indicating the percentage of viable green cells, apoptotic green cells, non-viable red cells and cells colored green and red after a 24-hour incubation period

Concentration (□ M)	Percentage (%)		
	Viable	Apoptotic	Nonviable
DMSO Control	88	7	5
J2	70	16	14
J5	64	17	19
J10	59	20	21
J20	51	23	26
J50	42	24	34
J100	36	27	37

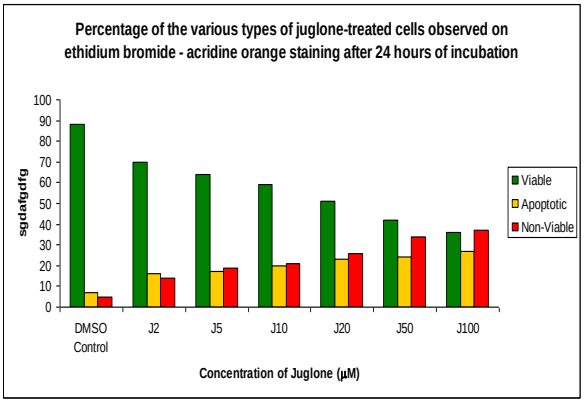


Figure 16: Graphical representation of the Ethidium Bromide-Acridine Orange dual staining results of cells treated with juglone for 24 hours

Table 7: Percentages of the various types of juglone-treated cells at 48 hours, observed by fluorescence microscopy when ethidium bromide-acridine orange dual staining method was employed

Concentration (□ M)	Percentage (%)		
	Viable	Apoptotic	Non-Viable
DMSO Control	72	19	9
J2	49	27	24
J5	43	28	29
J10	34	33	33
J20	26	34	40
J50	22	32	46
J100	19	31	50

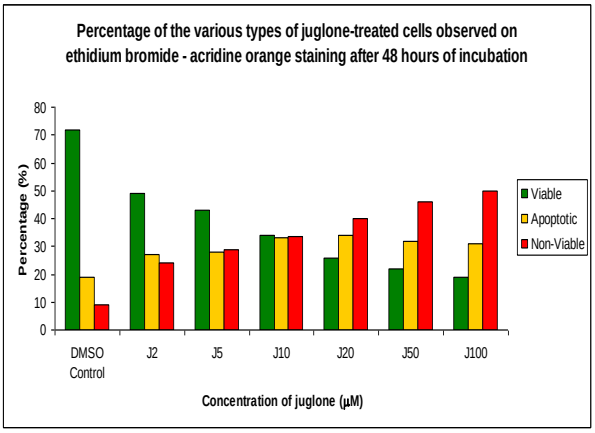


Figure 17: Graphical representation of the Ethidium Bromide-Acridine Orange dual staining results of cells treated with juglone for 48 hours

Table 8: Ethidium Bromide-Acridine Orange dual staining results indicating the percentage of viable green cells, apoptotic green cells, non-viable red cells and cells coloured green and red after 72 hours of incubation

Concentration (□ M)	Percentage (%)		
	Viable	Apoptotic	Non-Viable
DMSO Control	68	18	13
J2	42	24	34
J5	39	24	37
J10	25	30	45
J20	21	27	53
J50	19	23	58
J100	16	20	59

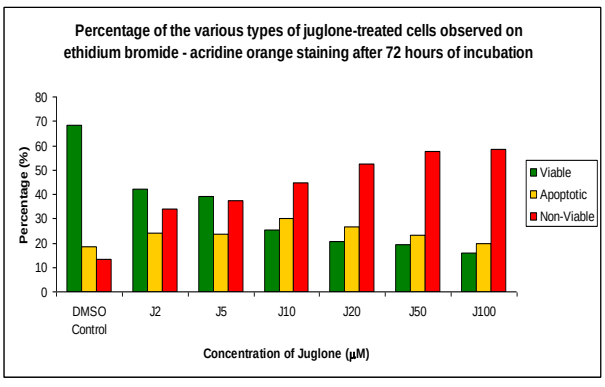


Figure 18: Graphical representation of the Ethidium Bromide-Acridine Orange dual staining results of cells treated with juglone for 72 hours

Detection of DNA Fragmentation by TUNEL assay

Human peripheral blood lymphocytes were cultures in varying concentrations of juglone for different time periods.DNA Fragmentation is one of the hallmarks of apoptosis. To study whether the juglone induced cytotoxicity is due to apoptosis, the cells are analysed by DNA fragmentation end-labelling method, or TUNEL assay. The principle of this assay lies in the fact that the Terminal deoxynucleotidyl transferase (TdT) binds to exposed 3'-OH ends of DNA fragments generated in response to apoptotic signals and catalyses the addition of biotin labelled and unlabelled deoxynucleotides. Diaminobenzidine(DAB) reacts with the labelled sample to generate an insoluble coloured substrate at the site of DNA fragmentation. Counterstaining with methyl green helps to morphological evaluate and characterize normal and apoptotic cells.

After incubating for 24 - 48 hours, nearly 98% of the control cells were found to stain green, indicating that they have not been stained by DAB (**Figures-19, 20**). This implies that the control cells had not undergone any DNA fragmentation, and hence apoptosis had not occurred. However, upon 24 hours of incubation, a significant number of the cells treated with concentrations of 10µM and above of juglone displayed brown colouration of part or whole of the cell, indicating DNA fragmentation, and hence, apoptosis. In **Figures(21,22)**, the increase in the apoptotic cells can be clearly noticed; these cells were treated to 20µM juglone for a period of 48 hours.

This indicates that with increasing concentration and incubation time, the cytotoxic effects of juglone on lymphocytes become more pronounced. Also, from the results of the TUNEL assay, we can conclude that cell death occurs by means of apoptosis.

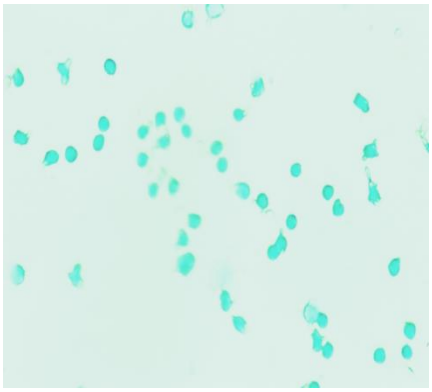


Figure 19: Control cells (48h) as seen after performing TUNEL assay. Note that all cells appear green in colour

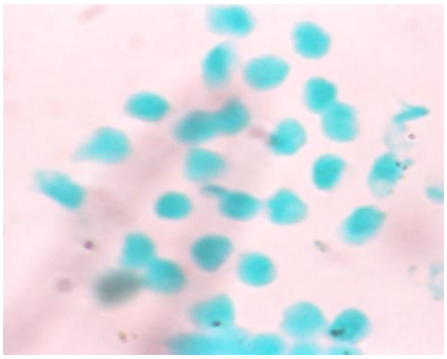


Figure 20: Cells after treatment with juglone (20µM) for 48 hours

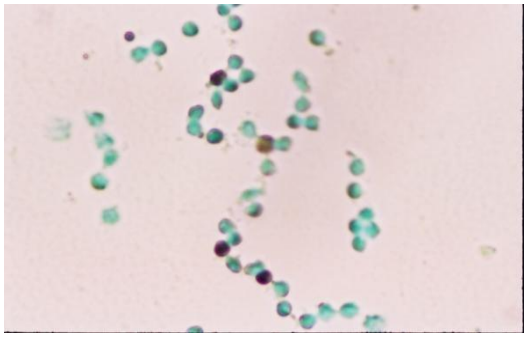


Figure 21:

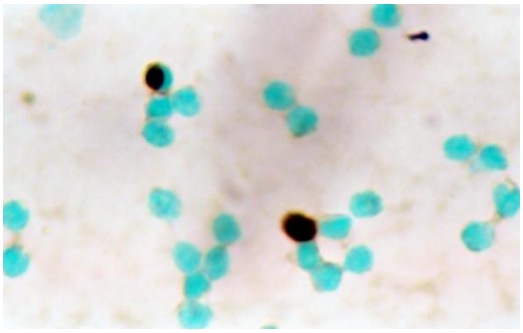


Figure 22: Green cells represent - Normal cells (No DNA Fragmentation)Brown cells represent - Cells with DNA fragmented nuclei

Evaluation of cytotoxic effects of juglone:

Further analysis of the signaling molecules responsible for the apoptotic cell death due to juglone treatment was attempted by performing Western blot studies. The juglone treated lymphocytes were analysed by performing immunoblotting for the Bcl-2 family of proteins like Bcl-2 and Bax, which are involved in response to apoptosis. Prior to performing Western blotting, Bradford assay and silver staining were carried out. However, this work is still in the preliminary stages and conclusions could not be drawn with the limited number of samples. It would, therefore, be ideal to conclude that a detailed study is necessary to study the signaling pathways of apoptosis. Our future goals include the immunochemical detection of proteins released in response to apoptosis and also determine the molecular mechanisms involved in the signalling pathways associated with the juglone induced apoptosis of human peripheral blood lymphocytes.

Figure23: An SDS gel after silver staining

DISCUSSION

The results obtained through trypan blue dye exclusion and MTT assay indicated that both plumbagin and juglone induce a concentrationand time-dependent cytotoxicity in resting human peripheral blood lymphocytes. The compounds also exhibit antiproliferative properties as observed from the reduction in proliferation of lymphocytes under the influence of PHA. The IC50 values of 6.5 and 11.6 μM after 24h of plumbagin and juglone treatment, respectively, obtained through MTT assay, indicate that plumbagin is about two times more toxic than

juglone. Although even 2 μMplumbagin is capable of inducing apoptosis in human resting lymphocytes, it has been reported that 5 μMplumbagin is not cytotoxic to resting mouse lymphocytes [12].

In another study [13] stated that Juglone also exerted similar concentration- and time-dependent toxicity in lymphocytes. At higher concentrations of plumbagin and juglone (20 and 50 μM), the viability values after treatment for 24 h were less than 40%, which fell to around 10% after 72 h treatment. The IC50 values obtained were 6.5, 3.8, and 1.8 μM for plumbagin at 24, 48, and 72 h, whereas for juglone the values were 11.6, 6.2, and 3.3 μM, respectively. From the IC50 values, plumbagin seems to be nearly two times more cytotoxic than juglone. Similar results were observed with trypan blue assay.

Jung-Il Chae, et al., [14] 2012 reported the effect of juglone on TPA-induced AKT, c-Jun, and c-fos activation in JB6 CI41 cells. Juglone inhibited TPA-induced (A) AKT, c-Jun, and (B) c-fos activation. JB6 CI41 cells were treated with juglone at 2.5 and 5 μM for 1 h before being treated with TPA (20 ng/ml) and harvested after 30 min. The protein expression levels of phosphorylated and total AKT, c-Jun and c-fos proteins were analyzed by western blot analysis. Equal loading of proteins was determined by incubating the same membrane with anti-β-actin antibody.

Very recently, it has been observed that juglone inhibits proliferation of human PBMCs with an IC50 value of 28 μM [15].

Priya Seshadri et al., 2011[16] reported that both plumbagin and juglone (2–10 μM) induce apoptosis in lymphocytes as inferred from the characteristic morphological features observed through optical, fluorescence, and electron microscopy. Quantitation through EtBr/AO staining indicates that a statistically significant percentage of cells show apoptotic features, whereas only about 10–15% of the cells undergo necrosis. Ultrastructural features of treated cells predominantly show condensed chromatin, crescent-shaped nuclei, nuclear fragmentation, and apoptotic bodies, thereby confirming an apoptotic mode of cell death. Uniformly distributed chromatin and well-preserved cellular organelles are seen in control lymphocytes. Progressive damage to lymphocytes is also witnessed through TEM. At lower concentrations (2 μM), chromatin marginalization is prevalent, and as concentration increases to 10 μM, a number of cells with heavily condensed chromatin, nuclear fragments, and apoptotic bodies are observed, indicating late stages of apoptosis. Apoptosis of human gastric cancer SGC-7901 cells due to juglone treatment.

Juglone has also been reported to induce apoptosis of human fibroblasts and melanoma cells, with a substantial increase in the percentage of cells in the sub-G1 region [17]. Fragmented patterns of DNA and occurrence of TUNEL positive cells obtained after plumbagin and juglone treatment further confirm apoptosis. A higher percentage of TUNEL-positive cells after plumbagin treatment also provide evidence for the higher Cytotoxicity of plumbagin compared to juglone.

CONCLUSION

From the present analysis, it is clear that juglone is toxic to human peripheral blood lymphocytes. The study of cytotoxity of juglone through viability studies like trypan blue dye exclusion method and MTT assay indicated that concentrations of 10μM and above decrease the percentage viability of lymphocytes significantly. Further, the analysis of the morphology, by means of Geimsa staining and ethidium bromide acridine orange dual staining (fluorescence microscorpy), of the treated cells is also in accordance with the viability tests. When TUNEL assay was performed, the percentage of cells with DNA fragmentation was found to increase greatly at higher concentrations (concentrations greater than 10μM). And since DNA fragmentation is an indication of apoptosis, the conclusion can be drawn that juglone causes cell death by means of apoptosis in human peripheral blood lymphocytes. However, further research is needed in this aspect, primarily for the immunochemical detection of the proteins released in response to apoptosis also to determine the molecular mechanisms involved in the signaling pathways, as this work is still in the preliminary stages and conclusions cannot be drawn with the limited number of samples used.

CONFLICT OF INTEREST: NO

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