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EVALUATION OF INVITRO CYTOTOXIC ACTIVITY OF PARTS OF *MURRAYA KOENIGII* (CURRY) PLANT ON HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMCS)

Sudeep Nagaraj[1]*, Jagadish Tavarekere Venkataravanappa[2] and Praveen Kumar Kondenahalli Subbarayappa[2]

[1]Sapthagiri Institute of Medical Sciences & Research Centre, Bangalore.

[2]Sri Devaraj Urs Academy of Higher Education and Research, Kolar.

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***Corresponding Author**
Sudeep Nagaraj
Sapthagiri Institute of Medical Sciences & Research Centre, Bangalore.

ABSTRACT

Murraya Koenigii (curry) extensively used in traditional medicine and it is reported to have several medicinal properties. It is also used in Indian cooking, However, *Murraya Koenigii* also reported to be toxic to some cell types. Human peripheral blood lymphocytes were treated with various concentrations of aqueous extracts leaf, fruit and stem to assess the toxic effects on the cells. Toxicity studies were performed by the Trypan Blue dye exclusion method. The decrease in viability percentage was noticed with increasing concentration of plant extracts in the cell culture system. Cell morphology was then studied using Acridine Orange and Ethidium Bromide staining and a significant increase in the apoptotic features were noticed in cells treated with the plant extracts. The apoptotic cell morphology has been evaluated by fluorescent microscopy. It was observed that exposure to increasing concentrations of crude plant extract (50mg/ml, 100mg/mL, and 150mg/mL) resulted in a concentration-dependent decrease in cell viability. Among the 03 parts of plant studied in the present work, lymphocytes treated with crude extracts of leaf showed highest cytotoxic and apoptotic activity.

KEYWORDS: Cytotoxicity; *Murraya Koenigii;* Acridine Orange; Ethidium Bromide; Trypan blue; Apoptosis.

INTRODUCTION

Treatment of cytotoxic agent to the normal cells can result in a variety of cell fates/structures. the cell may undergo many changes like necrosis in this cells lose its membrane integrity and becomes non-viable as a result of cell lysis. which will activate the genetics programme called cell death or apoptosis or it may stop the cell division.[1,2] Cells undergoing necrosis typically exhibit rapid swelling, loss of membrane integrity, shut down metabolism and release their contents into the environment.[1] Cells that undergo rapid necrosis in vitro do not have sufficient time or energy to activate apoptotic machinery and will not express apoptotic markers. Apoptosis is characterized by well-defined cytological and molecular events including a change in the refractive index of the cell, cytoplasmic shrinkage, nuclear condensation and cleavage of DNA into regularly sized fragments. Cells in a culture that are undergoing apoptosis eventually undergo secondary necrosis. They will shut down metabolism, fail membrane integrity and finally lyse.[2, 3]

Lymphocytes are the central cells of the immune system which are responsible for the maintaining the immune system the 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 a higher degree.[4] In the immune system, apoptosis is required for lymphocyte development and maintaining the homeostasis. Cell death of activated lymphocytes can result in autoimmune disorders and too much cell death can lead to immunodeficiency.[4]

Cell cytotoxicity refers to the ability of certain chemicals or mediator cells to destroy living cells. By using a cytotoxic compound, healthy living cells can either be induced to undergo necrosis (accidental cell death) or apoptosis (programmed cell death).[5] measurement of cytotoxicity has proved to be indispensable in the process of developing therapeutic anticancer drugs.[5]

There is an important role of medicinal plants for the development of cytotoxic agents, The medicinal use of plants is probably as old as mankind itself. Plants have continued to be a valuable source of natural products for maintaining human health, as studies on natural therapies have intensified. More than 150,000 plant species have been studied, and several of them contain therapeutic substances.[6,7] The use of plant compounds for the pharmaceutical purpose has gradually increased. According to the World Health Organization[8], medicinal plants are probably the best source of a variety of drugs. About 80% of individuals in

developed countries use traditional medicine containing compounds derived from medicinal plants.[9] The assessment of the cytotoxic potential of some of the medicinal plants is necessary to ensure relatively safe use of medicinal plants.[10,11]

MATERIALS AND METHODS

Collection of plant materials

In the present study, the plant evaluated was *Murraya Koenigii*. Fresh leaf fruit and stem were collected from Kolar area, shade-dried, coarsely powdered with a grinder and sieved. The finely powdered leaf material was stored at room temperature till further use.

Collection of peripheral blood and isolation of lymphocytes

Lymphocytes were obtained from the leftover blood samples after analysis from the central diagnostic laboratory SDUAHER, Tamaka, Kolar and Sapthagiri Institute of Medical Science and Research Center Bangalore. The cells were incubated at 37^0C for 72 h in 5 mL of culture medium consisting of 90% RPMI 1640 and 10% fetal bovine serum. Initial cell count was made by Haemocytometry. HiSep medium (an iso-osmotic, low viscosity medium containing polysucrose and sodium diatrizoate adjusted to a density of 1.0770±0.0010g/ml) was used for the isolation.

Subculturing of lymphocytes for the treatment

About 1mL of HiSep media was layered with 3mL of diluted blood and centrifuged at 800 rpm for 30 min at room temperature. The lymphocytes, which appeared as a buffy coat layer at the interface of HiSep Media and plasma, were aspirated and again centrifuged with phosphate buffered saline for purification. The lymphocytes were resuspended in 2 ml of RPMI 1640 cell culture medium. From this, 0.5 ml was transferred to four new cell culture tubes containing 4 ml of fresh RPMI 1640 media. Streptomycin (1%) and penicillin (1%) were added to inhibit the bacterial contamination. These tubes were kept in a CO_2 incubator at 37°C.

Preparation of plant materials

The dry powder of *Murraya Koenigii* leaf fruit and stem (5 g) was homogenized in 100 ml of distilled water in a 250 ml sterile conical flask. The mixture was then boiled for 60 minutes until the level of the aqueous solution becomes half. The extract was cooled to room temperature and filtered using Whatman No.2 filter paper and clarified by centrifuging at

5000 rpm for 30 min. The extract was stored in a refrigerator in order to be used for further experiments and then diluted to 50, 100 and 150 µg/ml in milliQ water.

Treatment of lymphocytes with plant extracts

The tubes containing lymphocytes were inoculated with 50, 100 and 150µg/ml of the plant extracts. One tube with lymphocytes and culture media was considered as control. All the treatments were performed in triplicate and the mean (± standard error) was calculated in each case. The tubes were labeled appropriately and kept in a CO_2 incubator at 37^0C for 24hr and monitored regularly.

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.[13] 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 color.

About 10 µl of cell suspension and 10 µl of trypan blue was taken in an Eppendorf, mixed and transferred 10 µl trypan blue treated cell solution onto a hemocytometer and observed under a microscope for viability count.

$$\text{PER CENT VIABILITY} = \frac{\text{NUMBER OF VIABLE CELLS}}{\text{TOTAL NUMBER OF CELLS}} \times 100$$

Assessment of cell morphology after plant extract treatment

Peripheral blood lymphocytes were cultured in varying concentrations of plant extracts and their morphology was studied using Fluorescence microscopy.

Fluorescence microscopy

Cell death can be studied morphologically by using differential Fluorescent dyes like Acridine Orange and Ethidium Bromide. Both control and varying concentration of all the three parts of plant extract treated lymphocytes were isolated from culture, about 100µl of cell suspension was taken and dropped on a clean glass slide and kept for drying on a slide warmer for 20 min. Then the slides were stained by using fluorescent dye at a concentration of 0.2% Acridine Orange and 0.2% Ethidium bromide respectively. The fluorescence emitted

and the morphology of the cells was observed in a fluorescence microscope using an appropriate filter.

RESULTS

Effect of plant extracts on cell viability

In the present study, Three parts of *Murraya Koenigii* plant were taken and assessed their cytotoxic effect on lymphocytes. The lymphocytes were treated with varying concentrations (50,100,150 mg/mL) of hot water extracts of the selected plant.

After 24 hrs of incubation, percent cell viability of both control and treated cells was carried out by using Trypan blue dye exclusion technique. The results show a dose-dependent response. All the three parts of plant extracts showed cytotoxic effects on lymphocytes in all the concentrations. The Effect of plant extracts on cell viability is illustrated in Figs. 1-4. The viability of lymphocytes treated with plant extracts fell as the concentration of the extracts increased.

The control sample showed 74.36% of viable cells and 25.64% non-viable cells after 24 hrs of incubation (Fig. 1).

Lymphocytes treated with extracts with 50mg/mL concentration were showed leaf extract 33% viable cells and 67% non-viable cells, Fruit extract 38.4% viable cells and 61.53% non-viable cells, Stem extract 37.38% viable cells and 53.27% non-viable cells, (Fig 2).

Lymphocytes treated with extracts with 100mg/mL concentration were showed leaf extract 31.77% viable cells and 68.22% non-viable cells, Fruit extract 32.22% viable cells and 67.77% non-viable cells, Stem extract 41.23% viable cells and 58.76% non-viable cells, (Fig 3).

Lymphocytes treated with extracts with 150mg/mL concentration were showed leaf extract 24.13% viable cells and 75.86% non-viable cells, Fruit extract 29.62% viable cells and 70.37% non-viable cells, Stem extract 38.75% viable cells and 61.25% non-viable cells, (Fig 4).

Trypan blue staining results of lymphocytes treated with Leaf, Fruit and Stem extracts with respective concentrations are shown in Table-1.

Morphological analysis using Fluorescent 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 the aberrant organization.

Acridine orange stain

Acridine orange is a fluorescent dye. It is used to visualize the number of cells which are undergoing apoptosis. Acridine orange can penetrate viable cells and stain them green. The morphology of the cells was observed in a fluorescence microscope using an appropriate filter.

Figures 5-8 show the number of viable cells in control, and the respective plant extract treated at 150mg/mL concentration. Control slide showed more number of viable cells since no extract has been added, whereas the treated cells with plant extract showed a significant reduction in a number of viable cells. Results were depicted in Table 2.

Percent viable cells were calculated by using the following formula.

$$\text{Percentage of viability} = \frac{\text{Total number of Acridine orange stained cells}}{\text{Total number of Acridine orange stained cells + ehidium bromide stained cells}} \times 100$$

Ethidium bromide staining

Ethidium bromide, which is excluded by viable cells, stains the nonviable cells and gives orange color. 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. Based on this concept, cells were stained with EtBr.

Compared to treated cells, in control slide, remarkably presence of less number of viable cells indicates that the control cells are more viable than the treated (Fig 9). On contrary, there were more non-viable cells observed in the cells treated with the plant extracts 150mg/mL (Fig 10-12). Results were depicted in Table 2.

Percent non-viable cells were calculated by using the following formula

$$\text{Percentage of non-viability} = \frac{\text{Total number of Ehidium bromide stained cells}}{\text{Total number of Acridine orange stained cells + Ehidium bromide stained cells}} \times 100$$

Percent of viable and non-viable cells

Results of Acridine orange and Ethidium bromide indicated that the cells treated with Leaf extract extracts showed 80.65% cytotoxic activity compared to Fruit, and Stem extract whose percentages are about 61.54% and 60.71% respectively shown in Table 2.

Table 1: Shows Trypan blue staining results of lymphocytes treated with Leaf, Fruit and Stem extracts.

Conc of the sample (µg)	Live cells	Dead cells	Total cells	Percent viable cells (%)	Percent non-viable cells (%)
Control					
	58	20	78	74.36 ±0.577	25.64 ± 1
Leaf extract					
50	34	69	104	33.00± 1.15	66.99± 1.15
100	34	73	107	31.77±1.15	68.22±1.73
150	28	88	108	24.13±1.15	75.86±1.15
Fruit extract					
50	40	64	104	38.4± 1.154	61.53±0.574
100	29	61	90	32.22±1.73	67.77±0.57
150	24	57	81	29.62±0.57	70.37±1.15
Stem extract					
50	40	57	97	37.38 ±1.15	53.27 ±1.15
100	37	51	88	41.23±1.15	58.76±1.15
150	31	49	80	38.75±1.15	61.25±1.15

Table 2: Shows Acridine orange and Ethidium Bromide staining results of lymphocytes treated with 150mg/uL of Leaf, Fruit and Stem extracts.

Plants used in the present study	No. Acridine orange stained cells	No. Ethidium bromide stained cells	Total number of cells	Percentage of viable cells	Percentage of non-viable cells
Control	11	7	18	61.11	38.88
Leaf extract	6	25	31	19.35	80.65
Fruit extract	10	16	26	38.46	61.54
Stem extract	11	17	28	39.28	60.71

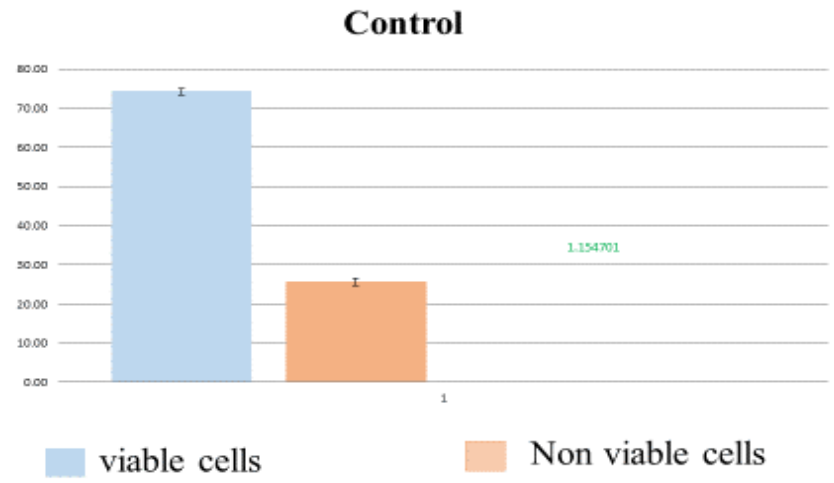

Fig. 1: Percent viability of lymphocytes of the control sample.

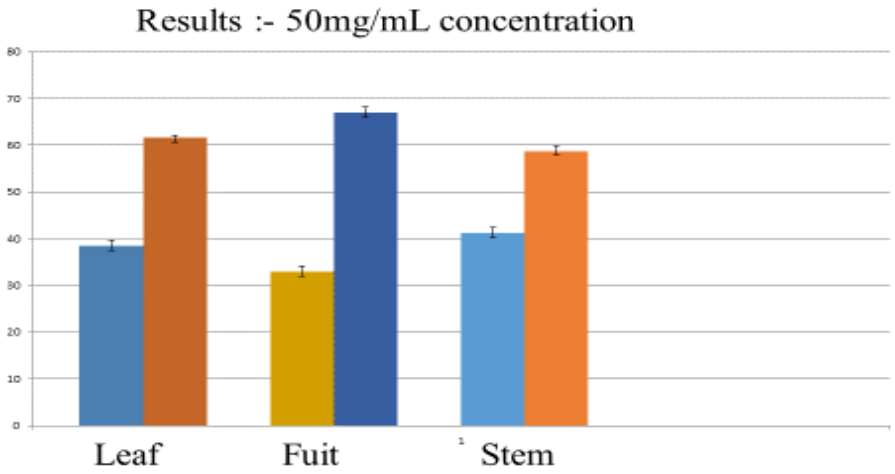

Fig. 2: Percent viability of lymphocytes treated with varying concentrations of 50mg/ml Extracts of leaf fruit and stem assessed by trypan blue dye exclusion method.

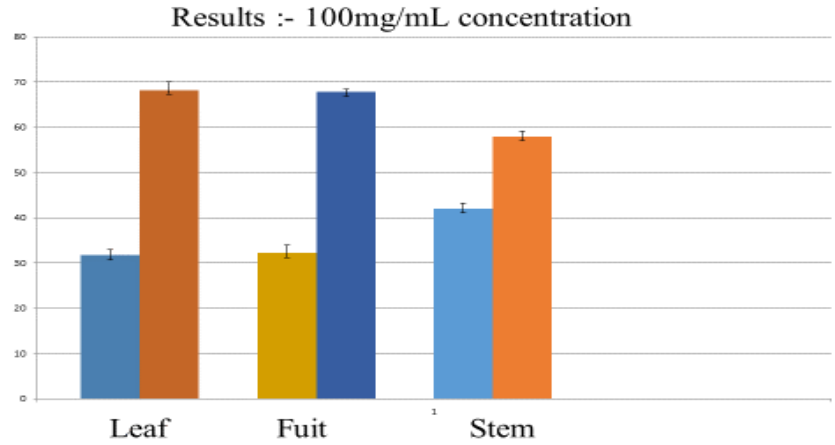

Fig. 3. Percent viability of lymphocytes treated with varying concentrations of 100mg/ml Extracts of leaf fruit and stem assessed by trypan blue dye exclusion method.

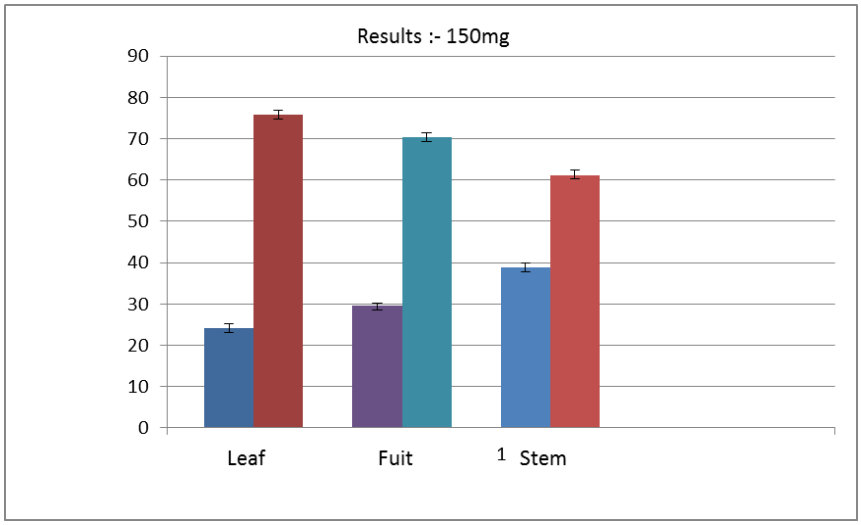

Fig. 4: Percent viability of lymphocytes treated with varying concentrations of 150mg/ml Extracts of leaf fruit and stem assessed by trypan blue dye exclusion method.

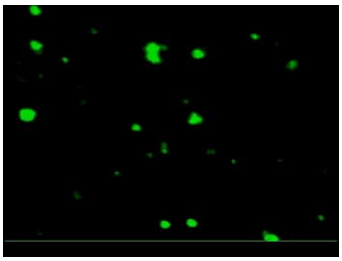

Fig. 5: Control cells stained with Acridine orange showing 10-11 viable cells.

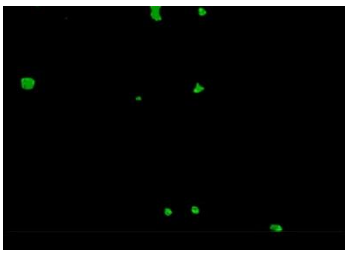

Fig. 6: Cells treated with Leaf extract stained with Acridine orange showing 5-6 viable cells.

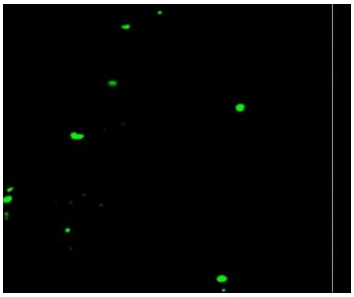

Fig. 7: Cells treated with Fruit extract stained with Acridine orange showing 9-10 viable cells.

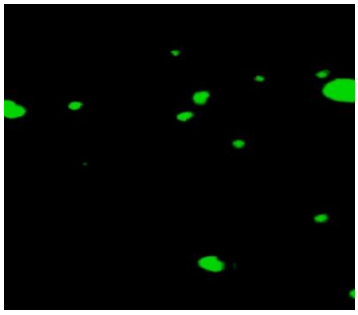

Fig. 8: Cells treated with Stem extract stained with Acridine orange showing 10-11 viable cells.

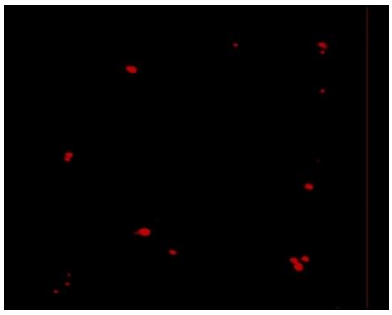

Fig. 9: Control cells stained with Ethidium bromide showing 6-7 non- viable cells.

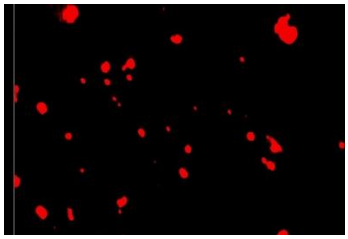

Fig. 10: Cells treated with Leaf extract stained with Ethidium bromide showing 24-25 non- viable cells.

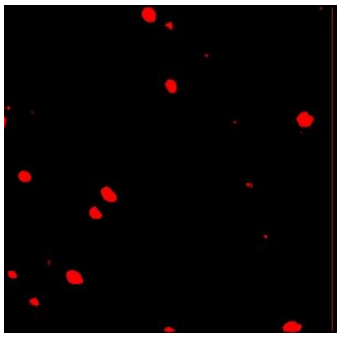

Fig. 11: Cells treated with Fruit extract stained with Ethidium bromide showing 15-16 non- viable cells.

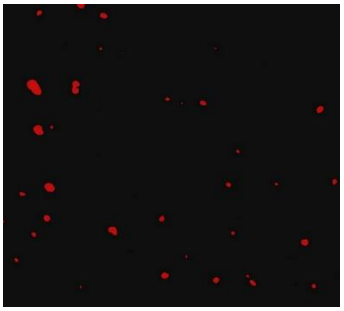

Fig. 12: Cells treated with Stem extract stained with Ethidium bromide showing 16-17 non- viable cells.

DISCUSSION

In the present study, we have demonstrated that three parts of plant *Murraya Koenigii* Leaf, Fruit, And Stem extracts have cytotoxic effects on cultured lymphocytes from a human. Among three extracts, we found that Leaf extracts showed the ability to inhibit cell survival. It is confirmed by three different methods: trypan blue dye exclusion assay, morphological observation of cells by two staining methods results showed that cell proliferation was inhibited at concentrations 50, 100 and 150 μg/mL.

One study showed that the leaf and stem could be used as an anticancer agent and it is a better cytotoxic agent.[17] In another study, High cytotoxicity of the CHCl3 extract of the root bark of M. koenigii have been demonstrated using different plant sources against different cancer cell lines have been reported.[19]

A study reported that Curry leaves have recently been found to be a potent antioxidant due to high concentrations of carbazoles, a water-soluble heterocyclic compound.[14] Another study showed that the presence of cytotoxic constituents and observed antibacterial and cytotoxic activities of *Murraya koenigii*.[15] In the other study, they isolated antioxidant protein from curry leaves, which exhibited a broad spectrum of antibacterial activity and suggested it as a promising candidate for a drug of an effective antioxidant antibiotic.[16]

CONCLUSION

From the present analysis, it is clear that Leaf, Fruit and Stem extracts of *Murraya Koenigii* were toxic to human peripheral blood lymphocytes. The study of cytotoxicity of these plant through viability studies like trypan blue dye exclusion method indicated that concentrations of 50mg/mL, 100mg/mL and 150mg/mL decrease the percentage viability of lymphocytes significantly. Further, the analysis of the morphology, by means of Ethidium bromide and

Acridine orange staining (fluorescence microscopy) of the treated cells is also in accordance with the viability tests.

The conclusion can be drawn that Leaf, Fruit and Stem extracts of *Murraya Koenigii* cause cell death by means of apoptosis in human peripheral blood lymphocytes. Comparatively, among the three plants parts studied, leaf extracts showed the highest cytotoxic activity than the other two parts of plant. However, further research is needed in this aspect, primarily for the immunochemical detection of the proteins released in response to apoptosis and also to determine the molecular mechanisms involved in the signaling pathways.

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