



## Evaluation of *In Vitro* Cytotoxic Effects of Three Medicinal Plants on Peripheral Blood Mononuclear Cells (PBMC)

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### ABSTRACT

*Annona squamosa*, *Datura metel*, and *Mentha piperita* are extensively used in traditional medicine. These were reported to have several medicinal properties. However, these plants are also reported to be toxic to some cell types. Human peripheral blood lymphocytes were treated with various concentrations of aqueous leaf extracts of all three plants to assess the toxic effects on the cells. Toxicity studies were performed by the Trypan Blue dye exclusion method. Decrease in viability percentage was noticed with increasing concentration of plant extracts in the cell culture system. Cell morphology was then studied using Giemsa staining and fluorescent microscope 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 (50 µg/ml, 100 µg/mL, and 150 µg/mL) resulted in a concentration dependent decrease in cell viability. Among the 03 medicinal plants studied in the present work, lymphocytes treated with *Annona squamosa* extract showed highest cytotoxic and apoptotic activity.

**Keywords:** Peripheral blood mononuclear cells; *Datura metel*; *Annona squamosa*; *Mentha piperita* L; Trypan blue; Acridine orange; Ethidium bromide; Giemsa stain

### INTRODUCTION

#### Cell Physiology

When normal cells are treated with any toxic compound, they may undergo necrosis due to lose of membrane integrity and rapid death occurs as a result of cell lysis. In this process, cells stop dividing and growing, or a decrease in cell viability, or can activate a genetic program to control cell death (apoptosis) [1]. Necrotic cells undergo rapid swelling, loss of membrane integrity, shut down metabolism and tend to release their contents into the surrounding medium [1]. *In vitro* necrotic cells do not have sufficient time or energy to activate apoptotic machinery. The cytological and molecular events when a cell becomes apoptotic are a change in the refractive index, shrinkage of the cytoplasm, nuclear condensation and DNA fragmentation. The apoptotic cells undergo secondary necrosis and lyse by shutting down the metabolism and by failure of membrane integrity [1].

#### The Lymphocytes

The lymphocytes are the central cells of the immune system and are responsible for adaptive immunity. In the human body, the lymphocytes continuously circulate in blood and lymph and are capable of migrating into tissue spaces and lymphoid organ [2].

Immune system should adopt apoptosis for lymphocyte development and homeostasis. The deregulation of lymphocyte apoptosis leads to a variety of immune disorders. There should be stable balance between these two processes in the immune system otherwise resulted either an autoimmune disorders or immunodeficiency [2].

### Cytotoxicity

The compounds that destroy live cells are the cytotoxic compounds. When cells are exposed to these compounds, they either undergo accidental cell death or programmed cell death [3]. Measuring cell cytotoxicity has a direct relation to measuring the efficacy of therapeutic anticancer drug [4,5]. Cytotoxicity is measured by determining the delay of proliferation of target cells either destructing the genetic material or by blocking the nutrient supply.

### Role of Medicinal Plants in Cytotoxicity

Since ages people started using the medicinal plants as therapeutics agents. Plants have invariable number of substances as natural products for its own benefit in tern proper maintenance of human health. Thousands of plant species have been identified as good source of therapeutics and use of these compounds is gradually increasing.

According to the World Health Organization [6] medicinal plant is probably the best source of a variety of drugs. In developed countries, about 80% of individuals use plant derived compounds. The use of plant extracts and phytochemicals can be of great significance in therapeutic treatments. Plants in general, synthesize toxic substances to protect themselves from infections, insects and herbivores. For safe use of therapeutics from medicinal plants, it is necessary to assess the cytotoxic potential of the compounds [7].

### Medicinal Properties of the Selected Medicinal Plants

*Annona squamosa*, commonly called as custard apple, is native of West Indies. It belongs to Annonaceae. The species is a small evergreen tree reaching 6-8 meters (20-26 ft.) tall, is commonly found in deciduous forests, cultivated throughout India and other countries. The plant is traditionally used for the treatment of epilepsy, dysentery, cardiac problem, worm infection, constipation, hemorrhage, antibacterial infection, dysuria, fever, and ulcer. It also has antifertility, antitumor properties [8-11]. The plant possesses potent bioactive principles in all its parts. Acetogenins, a class of natural compound, isolated from members of Annonaceae have potent anti-neoplastic, parasiticidal, pesticidal and anti-microbial activities [12-15]. Acetogenins belonging to a series of C-35/C-37, and derived from C-32/C-34 long chain fatty acids are known to be powerful inhibitors of complex I (NADH: ubiquinone oxidoreductase) in mammalian and insect mitochondrial electron transport systems [13,14]. Two Acetogenins, squamocin and squamostatin, isolated from *A. squamosa* have shown cytotoxic activity [15,16].

*Datura metel* belongs to Solanaceae. It is known for its toxicity also contains medicinal properties. *Datura* intoxication produces delirium (as contrasted to hallucination), hyperthermia, tachycardia, bizarre behavior, and severe mydriasis with resultant painful photophobia that can last for several days. *Datura* is known for its treatment against asthma. The active anti-asthmatic agent is atropine, which causes paralysis of the pulmonary branches of the lungs, eliminating the spasms that cause the asthma attacks. It is mentioned in Indian Ayurveda that the leaves are generally smoked either in a cigarette or a pipe [17,18]. Environmental factors play a major role on the concentrations of these compounds, so the toxicity also varies from plant to plant. The seeds are responsible for the anticholinergic toxicity of the plant [19]. *Mentha piperita* L, a perennial herb, belongs to Lamiaceae family. It is a cultivated as a sterile natural hybrid of *Mentha aquatica* (water mint) and *Mentha spicata* (spearmint). Although *M. piperita* is a native genus of the Mediterranean region, it has been spread all over the world for use in flavor, fragrance, medicinal and pharmaceutical applications [20-22]. The entire herb of *M. piperita* possesses antioxidant, cytotoxic, antiallergenic, antiviral and antibacterial activities [23]. The essential oil of *M. piperita* is reported to have antimicrobial and antioxidant activities [24]. Previous studies showed that *Mentha* has cytotoxic and anti-inflammatory activities [25]. Cytotoxicity assays are widely used by the pharmaceutical industry to screen for cytotoxicity in compound libraries. Researchers can either look for cytotoxic compounds, or they can screen "hits" from initial high-throughput drug screens for unwanted cytotoxic effects before investing in their development as a pharmaceutical.

## MATERIALS AND METHODS

### Collection of Plant Materials

Fresh leaves were collected from Kolar area and were authenticated by Dr. Amarananjundeshwara, Assistant Professor, (Vegetable Science), College of Horticulture, Kolar. Herbarium of all the three plants has been made and submitted to Dr. Madhava Chetty, Assistant Professor, Department of Botany, Sri Venkateswara Univerisy, Tirupati. Leaves were washed thoroughly, shade-dried, coarsely powdered in a blender and sieved. The finely powdered leaf material was stored at room temperature till further use.

### Collection of Peripheral Blood and Isolation of Lymphocytes

After getting ethics clearance (No. DMC/ KLR/ IEC/ 42/ 2016-'17) from the institute, left over blood was collected in Heparin vacutainers from the central diagnostic laboratory of Sri Devaraj Urs medical college. To about 1 mL of the blood added equal volume of cold PBS (pH 7.0). Mixed properly and carefully layered onto 2 mL Ficol in a falcon tube without getting mixed up, and spun down the contents at 3000 rpm for 30-45 min at room temperature. During the centrifugation the PBMCs moved from the plasma and were suspended in the density gradient, isolating them from erythrocytes and granulocytes. Removed plasma down to about 1cm above buffy coat, discarded, then aspirated buffy coat (the white layer lying on top of the red cells, it should come as one layer). The PBMC/buffy coat layer was washed twice with PBS. The PBS as supernatant was then removed and discarded. RPMI medium was prepared by mixing 40 mL of RPMI, 10 mL of FBS and 200  $\mu$ L Anitbiotic/antimycotic (Antibiotic antimycotic solution with Streptomycin 10 mg/20 mL, 10,000 U Penicillin, Amphotericin B and 0.9% normal saline). About 4mL of this mixer was dispensed in falcon tubes, added 30  $\mu$ L of PHA and 150-200  $\mu$ L of PBMCs and incubated with an atmosphere of 95% air and 5% CO<sub>2</sub> at 37°C for 4 hours [26].

### Preparation of Plant Materials

The dry leaf powder of the selected plants (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 kept at 65°C till no moisture is seen. The dried material was scraped and stored in a refrigerator in order to be used for further experiments. About 1mg of the dried extract was dissolved in 1 mL of distilled water and this served as stock.

### Treatment of Lymphocytes with Plant Extracts

After 4 hours of incubation, the lymphocytes were inoculated with 50, 100 and 150  $\mu$ g/ml of the plant extracts from 1 mg/mL stock. Lymphocytes in culture media without plant extract were considered as control. All the treatments were performed in triplicate and the mean ( $\pm$  standard error) was calculated in each case. The flasks were labeled appropriately and kept in a CO<sub>2</sub> incubator at 37°C for 24 hr 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 [27]. 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.

About 10  $\mu$ l of cell suspension and 10  $\mu$ l of trypan blue were taken in an eppendorf, mixed, left for ten minutes at room temperature. Transferred 10  $\mu$ l trypan blue treated cell solution onto haemocytometer, and observed under 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

#### 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 [28]. Both control and varying concentrations of all the three plant extract treated lymphocytes were isolated from culture. About 100  $\mu$ l of cell suspension was taken and dropped on a clean glass slide and kept for drying on slide warmer for 20 min. Then the slides are stained by using Giemsa stain for 2 min and washed two times with distilled water and morphology of cells was observed under microscope.

#### Fluorescence microscopy:

About 100  $\mu$ l of cell suspension was taken and dropped on a clean glass slide and kept for drying on 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 [29].

### Statistical Analysis

Data are expressed as mean  $\pm$  standard deviation from three separate experiments.

## RESULTS

The PBMCs were treated with 50, 100 and 150  $\mu\text{g/ml}$  of plant extract. After 24 hrs of incubation, per cent cell viability of both control and treated cells was carried out by using trypan blue dye exclusion technique. The results showed dose dependent response. All the three plant extracts showed cytotoxic effects on lymphocytes in all the concentrations. The viability of lymphocytes treated with plant extracts fell down as the concentration of the extracts increased (Figures 1-5).

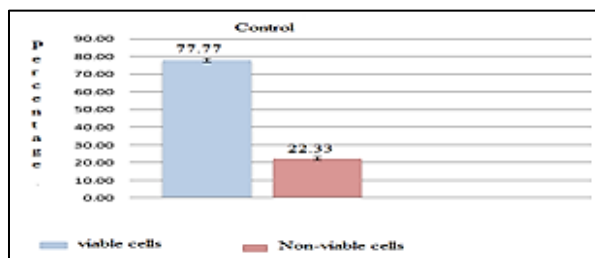


Figure 1: Percent viability of lymphocytes of control sample

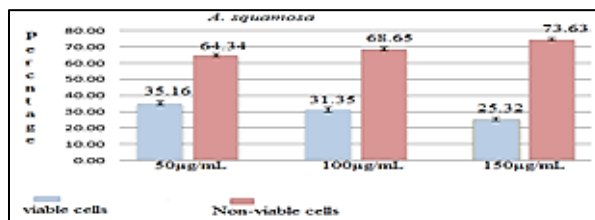


Figure 2: Percent viability of lymphocytes treated with varying concentrations of the extract assessed by trypan blue dye exclusion method

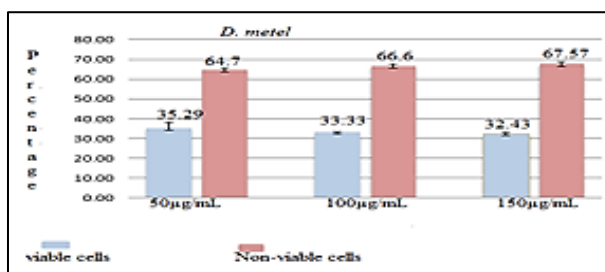


Figure 3: Percent viability of lymphocytes treated with varying concentrations of the extract assessed by trypan blue dye exclusion method

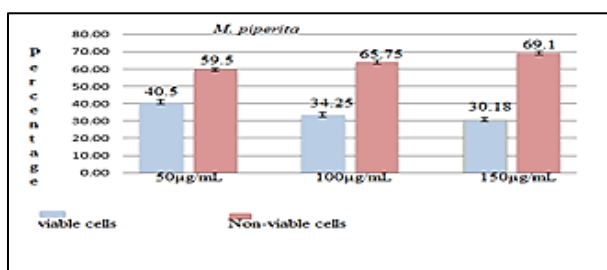


Figure 4: Percent viability of lymphocytes treated with varying concentrations of the extract assessed by trypan blue dye exclusion method

After 24 hours, in control 77.77% live cells and 22.23% dead cells were observed. The lymphocytes treated with *A. squamosa* leaf extract showed that 35.16% of viable cells and 64.83% of non-viable with 50 µg/mL, 31.35% viable and 68.65% non-viable with 100 µg/mL and 25.32% of viable cells and 73.68% of non-viable cells with 150 µg/mL of leaf extract. With *D. metel* leaf extract, 35.29% of viable cells and 64.71% of non-viable with 50 µg/mL, 33.33% viable cells and 66.67% viable cells with 100 µg/mL and 32.43% of viable cells and 67% of non-viable cells with 150 µg/mL and with *M. piperita* leaf extract, it was 40.5% of viable cells and 59.5% of non-viable with 50 µg/mL, 34.25% viable cells and 65.7% non-viable cells with 100 µg/mL and 30.88% of viable cells and 69.12% of non-viable cells with 150 µg/mL (Table 1).

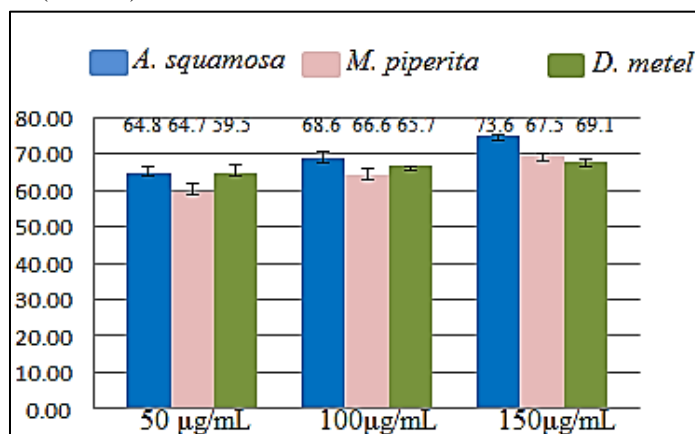


Figure 5: Viability comparison analysis of lymphocytes with all concentrations of plant extracts

Table 1: Per cent viable and non-viable cells when treated with different concentrations of plant extracts. Values are expressed as mean  $\pm$  S.E. (standard error)

Conc of the sample (µg)	Live cells	Dead cells	Total cells	Per cent viable cells (%)	Per cent non-viable cells (%)
<i>A. squamosa</i>					
50	32	59	91	35.16 $\pm$ 1.1	64.84 $\pm$ 1.7
100	37	81	118	31.35 $\pm$ 1.1	68.65 $\pm$ 1.7
150	19	56	75	25.32 $\pm$ 1.1	73.68 $\pm$ 0.5
<i>M. piperita</i>					
50	32	47	79	40.5 $\pm$ 1.7	59.5 $\pm$ 2.3
100	25	48	73	34.25 $\pm$ 1.7	65.75 $\pm$ 1.7
150	21	47	68	30.88 $\pm$ 1.1	69.12 $\pm$ 1.1
<i>D. metel</i>					
50	36	66	102	35.29 $\pm$ 2.8	64.71 $\pm$ 2.3
100	28	56	84	33.33 $\pm$ 0.01	66.67 $\pm$ 0.01
150	24	50	74	32.43 $\pm$ 0.5	67.57 $\pm$ 1.1
Control	56	16	72	77.77 $\pm$ 1.1	22.23 $\pm$ 1.1

The results of the present experiment showed that out of three plant extracts, *A. squamosa* showed more cytotoxic activity on lymphocytes with all the concentrations. *D. metel* and *M. piperita* showed almost similar cytotoxic effect on lymphocytes. At the highest concentration tested (150 µg/mL) with a 24 hour incubation period, *A. squamosa* leaf extract resulted in 73.68% (on an average) inhibition of lymphocyte proliferation.

### Morphological Analysis

#### Giemsa stain:

The lymphocytes were collected from culture treated with varying concentrations of all the three plant extracts and control and stained by Giemsa. Figure 6 showed the morphology of the control cells. There was a significant alteration in cell membrane blebbing, loss of cell death through apoptosis in control cells. The distinct apoptotic features are indicated by arrows. The morphology of the cells treated with *A. squamosa* (Figure 7), *D. metel* (Figure 8), *M. piperita* (Figure 9) showed significant alterations in cell shrinkage, cell membrane blebbing, changes to the cell membrane such as loss of membrane asymmetry and attachment, cell shrinkage. Arrows indicate the distinct apoptotic feature seen in cells.

**Fluorescent microscopy:**

Figures 10-13 showed the number of viable cells in control, and the respective plant extract. 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 number of viable cells. Results were depicted in Table 2 with EtBr, compared to treated cells, control slide showed remarkably less number of cells. This indicates that the control cells are more viable than the treated (Figure 14). On the contrary, there were more non-viable cells observed in the cells treated with the plant extracts (Figures 15-17). Results were depicted in Table 2.

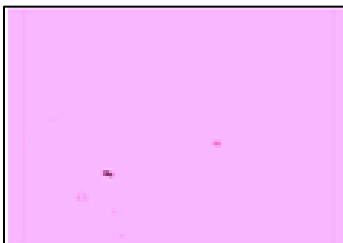


Figure 6: Control cells stained with Giemsa after an incubation of 24 hours

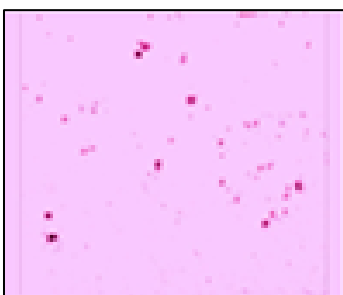


Figure 7: Cells were stained with Giemsa after 24 hours of incubation with *A. sqamosa*

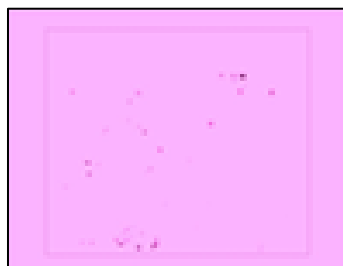


Figure 8: Cells were stained with Giemsa after 24 hours of incubation with *D. metel*

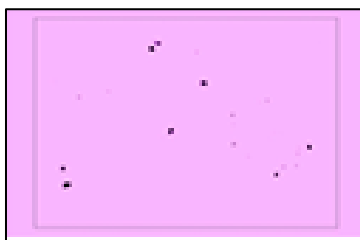


Figure 9: Cells treated with *M. piperita* and stained with Giemsa after an incubation of 24 hours

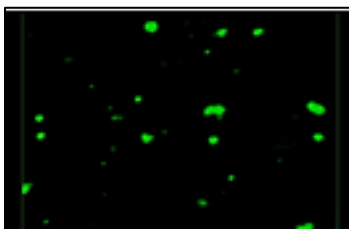


Figure 10: Control cells stained with Acridine orange showing 16, 17 viable cells

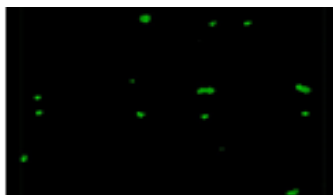


Figure 11: Control cells treated with *A. sqamosa* stained with acridine orange showing 4, 5 viable cells

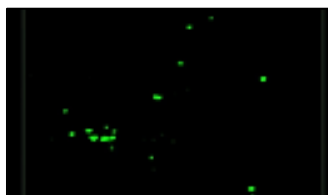


Figure 12: Control cells treated with *D. metel* stained with acridine orange showing 8, 9 viable cells

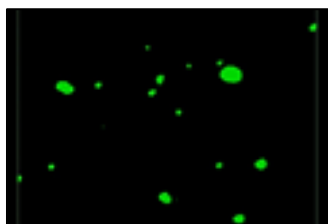


Figure 13: Control cells treated with *M. piperita* stained with acridine orange showing 9, 10 viable cells

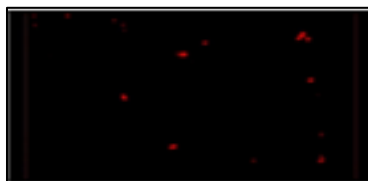


Figure 14: Controlled cells stained with ethidium bromide showing 4, 5 non- viable cells

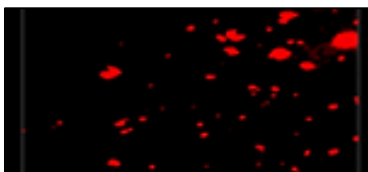


Figure 15: Cells treated with *A. sqamosa* stained with ethidium bromide showing 19, 20 non-viable cells

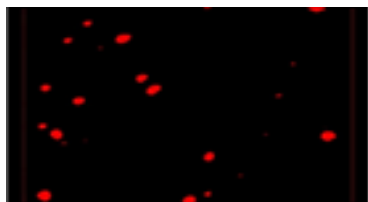


Figure 16: Cells treated with *D. metel* stained with ethidium bromide showing 17, 18 non-viable cells



Figure 17: Cells treated with *M. piperita* stained with ethidium bromide showing 18, 19 non-viable cells

Table 2: Percent viable and non-viable cells when stained with acridine orange and ethidium bromide under fluorescent microscope

Plants used in the present study	No. Acridine orange stained cells	No. Ethidium bromide stained cells	Total number of cells	Per cent viable cells (%)	Per cent non-viable cells (%)
Control	17	5	22	77.27	22.73
<i>A. squamosa</i>	5	20	25	20	80
<i>D. metel</i>	9	18	27	33.33	66.67
<i>M. piperita</i>	10	19	29	34.48	65.52

The results indicated that the cells treated with *A. squamosa* extract showed 80% cytotoxic activity compare to *D. metel*, and *M. piperita* whose percentages were about 66.67% and 65.65% respectively.

## DISCUSSION

In the present study, we have demonstrated that all three plants *A. squamosa*, *D. metel*, and *M. piperita* extracts have cytotoxic effects on cultured lymphocytes from human. Among three extracts, we found that *A. squamosa* showed the ability to inhibit cell survival. It is confirmed by three different methods: trypan blue dye exclusion assay, morphological observation of cells by three staining methods. Previous studies showed that the hot water extract of *A. squamosa* leaf has significant hypoglycemic and thus anti-diabetic activity in experimental animals [30] and seeds of plant have showed remarkable anti-microbial and cytotoxic activities [31], whereas ethanolic extract of leaf and stem has reported anticancer [32]. The *Annona* sp. is a promising source of potential compounds exhibiting cytotoxic activities against PBMC and our investigation showed good result on PBMC. Further research is needed to identify active antiproliferative drug from aqueous extract of *A. squamosa*. In our study, *D. metel* leaf aqueous extract showed moderately cytotoxic effect on lymphocytes. This is in agreement with Soumen Roy's work on evaluation of *in vitro* cytotoxic and antioxidant activity of *D. metel* and *Cynodon dactylon* extracts [33]. The cytotoxicity of Labiatae family including the *Mentha* genus has been reported. Moreover, the *Mentha* preparations have been used for therapy of human cervical cancer [34]. The cytotoxic effect of essential oil of *M. pulegium* on ovarian adenocarcinoma (SK-OV-3), human malignant cervix carcinoma (Hela) and human lung carcinoma (A549) cell lines has been shown by Shirazi et al. In this study methanolic extract at a concentration of 1000 µg/ml did not show any cytotoxic effect [35]. On the other hand, in our study, there was gradual increase in cytotoxicity from 50-150 mg/mL, which is 50 times greater than that in Shirazi's study in terms of concentration used. Furthermore, they used clonogenic and neutral red (NR) assays for assessment of cytotoxicity, while we studied the effect of aqueous extract of *M. piperita* and used trypan blue dye exclusion for evaluation of its cytotoxicity.

## CONCLUSION

From the present analysis, it is clear that *A. squamosa*, *D. metel* and *M. piperita* were toxic to human peripheral blood lymphocytes. The study of cytotoxicity of these plants through viability studies like trypan blue dye exclusion method indicated that concentrations of 50 mg/mL, 100 mg/mL and 150 mg/mL decrease the percentage viability of lymphocytes significantly. Further, data obtained using the method of morphology analysis, by Geimsa staining and Ethidium bromide, as well as the fluorescence microscopy of Acridine orange stained cells are in agreement with the viability tests. We conclude that *A. squamosa*, *D. metel* and *M. piperita* cause cell death by means of apoptosis in human peripheral blood lymphocytes to varying degrees. Comparatively, among the three plants studied, *A. squamosa* showed highest cytotoxic activity than the other two plants. However, further research is needed in this aspect, to identify the antileukemic agents and their action on cell lines, 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.



## ACKNOWLEDGEMENTS

Authors are grateful to Sri Devaraj Urs Academy of Higher Education and Research for providing the materials required for completion of the work. We acknowledge institution ethics committee (No. DMC/ KLR/ IEC/ 42/ 2016-'17) to provide us clearance to start the work and publish.

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