Review Article
ISSN: 2249-1236
CODEN: IJRRBI (USA)
Iswariya *et al.*, *Int.* J. Res. Rev. Pharm. Appl. Sci.,*(2018) 8(1) 055-068*
International Journal of Research and Reviews in Pharmacy and Applied sciences

In Vitro evaluation of Antileukemic activity of the methanol extract of *Citrus Limetta, Citrus Sinensis* pulp and Naringenin

Author(s)& Affiliation
ISWARIYA. G.T, PADMA, P.R AND NIRMALADEVI, R*
Department of Biochemistry, Biotechnology and Bioinformatics, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore, TamilNadu-641 043

Corresponding Author:

ISWARIYA. G.T

Abstract:

Objective: The present study was framed to assess the antileukemic activity of the methanol extract of *Citrus limetta, Citrus sinensis* pulp extracts and naringenin.

Methods: The antileukemic activity of the citrus fruit pulp extracts and naringenin were determined by the MTT and SRB assay in Molt-3 leukemic cells and normal PBL. The apoptosis inducing ability of the extracts and naringenin were studied by Giemsa and AO/EtBr staining which was further confirmed by flow cytometric analysis (Annexin V/FITC and Jc-1 staining).

Results: The results demonstrated that both the citrus pulp extracts and naringenin were able to induce cell death in Molt-3 cells but were not toxic to normal PBL. The results confirmed that the citrus fruit pulp extracts and naringenin induced cell death via apoptosis.

Conclusion: The citrus fruit pulp extracts and naringenin were able to induce cell death in Molt-3 cells via intrinsic apoptotic pathway and also exhibited differential response against Molt-3 cells and PBL.

Keywords: *Citrus limetta*, *Citrus sinensis*, naringenin, Molt-3, PBL, antileukemic activity

Introduction:

Leukemia is a group of malignant diseases originating from blood or bone marrow cells which includes Chronic Myeloid Leukemia (CML), Acute Myeloid Leukemia (AML), Chronic Lymphoblastic Leukemia (CLL) and Acute Lymphoblastic Leukemia (ALL) [1]. In India, acute lymphoblastic leukemia (ALL) is mainly childhood disease that arises from recurrent genetic alterations which block precursor B- and T-cell differentiation and drive aberrant cell proliferation and survival. It is characterized by the accumulation of malignant, immature lymphoid cells in the bone marrow and also in peripheral blood. The incidence of ALL represents about 80% of all childhood leukemia and peaks from 2 to 5 years of age, while it is relatively rare in adults [2,3].

The major disadvantages with chemotherapeutic agents are severe and adverse side effects and multi-drug resistance. Some reasons by which cancer cells become resistant to therapies are drug efflux systems, amplification of drug targets, or changes in drug kinetics. The adverse effects of cancer chemotherapy can be treated symptomatically, but in some cases such secondary treatments may become very toxic, which is not acceptable to some cancer patients. Due to the disadvantages associated with conventional cancer chemotherapies and the advantages of more natural treatment options, there has been a growing interest in the use of complementary and alternative medicines [4].

Citrus belongs to the family *Rutaceae* which comprises about 40 species; mainly distributed in Brazil, China, India, Mexico, United States, Spain and Pakistan [5]. *Citrus limetta*, sweet lime (Mousambi) is a popular indigenous fruit relished for its cooling and therapeutic effects. Medicinal value of *Citrus limetta* is attributed by the presence of various compounds which act as the potential sources of anticancer, antimicrobial, antioxidant, anti-inflammatory and antithrombotic action [6]. *Citrus sinensis* or sweet orange originated from south East Asia and has been used traditionally to treat ailments like constipation, cramps, diarrhoea, bronchitis, tuberculosis, cough, cold, obesity, menstrual disorder, angina, hypertension, anxiety, depression and stress [7]. Naringenin is a common dietary flavonoid abundantly present in fruits and vegetables, is readily formed from naringin after dietary intake in humans. It is also present in the herbs (approximately 10–15%). Naringenin has a variety of pharmacological functions, such as antioxidant activity, anti-inflammatory activities, modulation of hepatic apolipoprotein and lipid synthesis, and antimutagenic effect [8]. With this background, the present study aimed to evaluate the antileukemic potential of methanol extract of *Citrus limetta*, *Citrus sinensis* pulp and commercially available compound naringenin using leukemic cell line (Molt-3) in comparison with the normal peripheral blood lymphocytes.

Materials and Methods:

Preparation of *Citrus limetta* and *Citrus sinensis* extracts:

Fresh fruits (*Citrus limetta* and *Citrus sinensis*) were procured from local markets in Coimbatore, India. The peels and pulp were separated. The pulp was sliced into small pieces and kept in an incubator for 12 hours at 40°C. About 20g of pulp was weighed and mixed with 100ml of methanol and kept overnight in a shaker incubator. The extracts obtained were filtered, concentrated and different concentrations ranging from 10µg to 200µg used for *in vitro* assays. Various concentrations of naringenin (MP Biomedicals, USA) ranging from 10µM to 200µM were prepared by diluting 1M stock and were used for *in vitro* anticancer assays.

Culturing of Molt-3 cell line and peripheral blood lymphocytes:

Molt-3 T-cell Acute Lymphoblastic Leukemic cell line, was purchased from NCCS, Pune, India. It is originally derived from 19 year old male with acute lymphoblastic leukemia. It was cultured using RPMI 1640 medium supplemented with 10% FBS and incubated at 37˚C. The cell count and viability was tested with trypan blue using haemocytometer and 1×10^6 cells were seeded onto 96 and 6 well plates and were treated with extracts for 24 hour and then cytotoxic and apoptotic assays were performed. Etoposide is well-known to trigger caspase-mediated apoptosis and also targets topoisomerase II thus leading to the production of DNA breaks and eliciting a response that affects several aspects of cell metabolism [9]. Hence, in the present study it is used to compare the anticancer activity of the citrus fruit extract and naringenin.

Fresh blood was drawn from healthy individual (the human ethical clearance no: AUW/IHEC/BC-16-17/XMT-01) by vein puncture under aseptic conditions, using heparinized syringe. The blood was then diluted 1:1 with RPMI 1640 medium. About 4ml of lymphosep (MP biomedicals) was dispensed into centrifuge tube. About 2ml of the diluted blood was carefully layered on top of the lymphosep medium and centrifuged for 30minutes at 400Xg at 18-20°C. The lymphocytes were separated and washed with PBS twice. Then the cells were resuspended in RPMI 1640 medium supplemented with 10% FBS.

MTT dye reduction assay:

MTT (-3-(4,5-dimethylthiazol- 2-yl)- 2,5-diphenyltetrazolium bromide) assay was performed as per Igarashi and Miyazawa, (2001) [10]. The treated Molt-3 cells and Peripheral Blood Lymphocytes (PBL) were incubated with 50µl of MTT at 37ºC for 3 hours after centrifugation. After incubation, 200µl of PBS was added to all samples. The liquid was then carefully aspirated. Then 200µl of acid propanol was added and left overnight in the dark. The absorbance was read at 650nm in a micro titer plate reader (Anthos 2020, Austria). The optical density of the control cells were fixed to be 100% viable and the per cent viablity of the cells in the different treatment groups were then calculated using

Per cent cell viability = $[(C- T)] / (C)] \times100$

Where, T- Absorbance of test sample; C - Absorbance of control

SRB assay:

Sulphorhodamine B (SRB) assay was carried out as per Skehan *et al*. (1990) [11]. The treated Molt-3 cells and PBL were collected by centrifugation and washed with PBS. An aliquot of 350µl of ice-cold 40% TCA was layered on the top of the treated cells and incubated at 4ºC for one hour and then washed 5 times with 200µl of ice cold PBS. The PBS was removed and SRB dye (350µl) was added to each tube and left in contact with the cells for 30 minutes at room temperature. After which they were washed 4 times with 1ml portion of 1% acetic acid to remove the unbound dye, then 350µl of 10mM Tris (pH 10.5) was added to each tube to stabilize the protein bound dye. The pellet was shaken gently for 20 minutes on a gyratory shaker. The debris was spun down and the absorbance of the tris layer in each group was transferred to a 96-well plate and read in a microtiter plate reader at 490nm. The per cent viability of the cells in the treatment groups were calculated using

Per cent cell viability = $[(C- T)] / (C)] \times100$

Where, T- Absorbance of test sample; C - Absorbance of control

Giemsa staining:

The various morphological characteristics of apoptosis that may occur were observed in ALL cells and PBL in the presence and absence of the methanol fruit pulp extracts compared with the standard chemotherapeutic drug etoposide. Giemsa staining was performed as per protocol explained by Chin *et al*. (2001) [12]. After the treatment with the extracts, Molt-3 cells were spun down and the pellet formed was resuspended in PBS and spread on microscopic slides with 10µl of diluted Giemsa stain and observed using a phase contrast microscope (Nikon, Japan) at 400X magnification. The number of apoptotic cells and normal cells were counted. The apoptotic ratio can be calculated by the following formula,

Apoptotic ratio = Number of apoptotic cells/Number of normal cells

Acridine orange/Ethidium bromide (AO/EtBr) staining:

AO/EtBr staining technique is used to differentiate between quiescent and actively proliferating cells and also used to measure apoptosis. The method adopted for the study is as elucidated by Parks *et al*. (1979) [13]. The treated cells were spun down at 1500rpm for 10 minutes and the supernatant was discarded. AO/EtBr (10µL) was added to the treated cells and spread by placing cover slip over it. The stained slides were incubated at room temperature for 5 minutes. The apoptotic cells with condensed chromatin and fragmented nuclei were identified by red fluorescence and the normal cells were visualized by green fluorescence which was counted using an upright fluorescent microscope using B2A filter at 400X magnification. The apoptotic ratio was calculated as,

Apoptotic ratio = Number of apoptotic cells/ Number of normal cells

Determination of apoptosis:

Induction of apoptosis elucidated by the extracts were determined using Annexin V/FITC Apoptosis detection kit (BD Biosciences). The procedure was followed according to the manufacturer protocol and the stages of apoptosis were identified using flow cytometer (BD Facsverse).

Determination of mitochondrial membrane potential:

Mitochondria play a critical role in apoptosis by releasing cytochrome c and other proteins that are essential for the activation of pro-caspase-9 and the execution of apoptosis which is measured using BD mitoscreen (JC-1) kit (BD biosciences). The procedure was followed according to the manufacturer protocol and mitochondrial membrane potential were assessed using flow cytometer (BD Facsverse).

Results and Discussion:

MTT assay:

In order to determine the cytotoxic effect of the methanol extract of *Citrus limetta*, *Citrus sinensis* pulp and naringenin, MTT assay was performed in leukemic cell line (Molt-3) which was compared with the normal peripheral blood lymphocytes. The experiment was carried out using varying concentrations of citrus fruit pulp extracts ranging from 10, 25, 50, 100 and 200µg and naringenin ranging from 10, 25, 50, 100 and 200µM. The results indicate that the citrus fruit pulp extracts and naringenin did not induce leukemic cell death at a lower concentration, while, a decline in cell viability was observed when co-administered with etoposide indicating that along with the extracts, naringenin could augment the cytotoxic effect of etoposide in Molt-3 leukemic cell line. The results are provided in Figure 1.

The cytotoxic effect of the citrus fruit pulp extracts and naringenin were tested against the normal peripheral blood lymphocytes and the results showed that at lower concentration of 10µg and 10µM the percent viability were found to 77%, 78% and 70% for *Citrus limetta* and *Citrus sinensis* pulp and naringenin which was increased further with the increasing concentration. Among the extracts tested, *Citrus sinensis* pulp extract showed less toxicity than the *Citrus limetta* pulp extract and naringenin respectively. When normal peripheral blood lymphocytes were treated with etoposide, the viability was reduced to a greater extent. In order to determine the protective effect of the citrus fruit pulp extract and naringenin, PBL were co-treated etoposide at a concentration of 200µM. The viability were increased in both the extracts and naringenin after treating with the etoposide. Thus, the results clearly indicate the differential response of the citrus fruit pulp extracts and naringenin against Molt-3 leukemic cells and normal peripheral blood lymphocytes.

SRB assay:

In SRB assay, the results demonstrated that all the three treatment groups exhibited significant cytotoxic effect against Molt-3 leukemia cell line. When Molt-3 cells are treated in combination (etoposide along with fruit pulp extracts and naringenin), cell death was induced even at a lower concentration. But at higher concentration, there is not much significant difference in the level of cell death when etoposide is treated along with the pulp extracts. But at higher concentration, it was found that pulp extracts and naringenin alone treated group causing significant leukemic cell death. The results are provided in Figure 2.

In the present study, the normal peripheral blood lymphocytes were treated with varying concentrations of the extracts and naringenin and the results showed that the cell viability was found to increase with the increasing concentration. Further, treatment with etoposide caused a steep decrease in the cell survival rate and upon administration of the citrus fruit pulp extract and naringenin there is an improvement in the cell viability indicating its protecting activity in normal peripheral blood lymphocytes from cell death. The results showed that 50% viability was seen at 50µg and 50µM concentration. Hence, further analysis were carried out at 50µg and 50µM concentration of the extracts and naringenin.

The results of both the cytotoxic assays confirm that the methanol extract of *Citrus limetta* and *Citrus sinensis* and naringenin were able to induce cell death in Molt-3 cell line while they are not causing toxic to normal PBL. However, the mode of cell death induced by the extracts is not known. Hence it becomes essential to analyze the mechanism of cell death induced by citrus fruit pulp extracts and naringenin.

Apoptosis is otherwise called programmed cell death occurring to discard the unwanted or dangerous cells. Commencing of apoptosis is identified by various morphological and nuclear changes such as cell shrinkage, chromatin condensation, nuclear fragmentation and membrane blebbing which can be observed by various staining techniques. In the present study, Giemsa and AO/EtBr staining technique were performed to identify the morphological and nuclear changes associated with apoptosis.

Giemsa staining:

The ALL cell line, Molt-3 and PBL were treated with the methanol extract of *Citrus limetta* and *Citrus sinensis* pulp and naringenin. The results demonstrated the reduction of cell volume, cell shrinkage, chromatin

condensation and cytoplasmic blebs in Molt-3 cell line while in PBL most of the cells remained intact. In molt-3 cell line, the number of apoptotic cells were increased with the increase in concentration of fruit pulp extracts and naringenin thus indicating its dose dependent activity. In contrast, upon treatment with the extracts and naringenin, the viability of the normal peripheral blood lymphocytes were increased. When the cells treated with extracts along with etoposide, the number of apoptotic cells were decreased which indicates the protective effect of the extracts against etoposide. Apoptotic ratio was calculated and tabulated as given in Table1 and 2.

AO/EtBr staining:

Acridine orange is a vital dye used in conjuction with ethidium bromide to differentiate between viable and apoptotic cells. It stains live cells with green color while ethidium bromide stains dead cells with red color. Ethidium bromide will stain only those cells that have lost their membrane integrity [14]. The results revealed that both the citrus fruit pulp extracts and naringenin increased the number of apoptotic cells in Molt-3 cells when compared to the normal PBL. When the cells were co-administered with the extracts and naringenin along with etoposide, the number of apoptotic cells were decreased even at lower concentration. The results showed that only few Molt-3 cells were stained green (live cells) while most of the cells were stained red (apoptotic cells) after the treatment with citrus pulp extracts as well as naringenin while in PBL, most of the cells were stained green indicating the differential response mediated by the citrus fruit extracts and naringenin. Thus, it is clearly evident that the pulp extracts and naringenin induce cell death in Molt-3 leukemic cells via apoptosis while rendering protection to the normal peripheral blood lymphocytes which were presented in Table 1 and 2. In order to confirm whether the citrus fruit pulp extracts and naringenin induce cell death by apoptosis, flow cytometric analysis were carried out.

Apoptosis is a mode of programmed cell death that is accompanied by numerous morphological as well as biochemical changes to the cellular architecture. Normally, phosphatidylserine resides in the inner plasma membrane of healthy cells but is rapidly externalised on the outer plasma membrane of cells exposed to pro-apoptotic stimuli. Phosphatidylserine exposure in response to apoptotic stimuli is a highly conserved evolutionary feature of mammalian and bacterial cells [15]. Thus, in the present study, the annexin V binding assay by flow cytometry was performed to detect apoptosis.

Annexin V-/FITC staining:

A fluorochrome-labelled Annexin V used in combination with a DNA-binding dye, such as propidium iodide (PI) differentiates between live cells (negative for both dyes), cells in early apoptosis (Annexin positive but PI negative) and dead cells (positive for both dyes) [16]. From the above results, the citrus fruit pulp extracts and naringenin were able to induce apoptosis in the Molt-3 cell line which was confirmed by Annexin V/FITC staining by flow cytometry. The results indicate that after treating with the citrus fruit pulp extracts and naringenin, most of the Molt-3 cells were in the late apoptotic stage of cell death and only very few cells were undergone early apoptosis. When treated along with standard drug etoposide, the percentage of apoptosis was reduced to some extent. When PBL was treated with the citrus fruit pulp extracts and naringenin, the number of apoptotic cells were found to be very low which indicates the non-toxic nature of the extracts as well as naringenin. Further observation revealed that citrus fruit pulp extracts at higher concentration exhibited better activity than the naringenin. Also a

slight decline in the activity was observed when administered along with etoposide. The results of annexin V/FITC staining reiterates the results observed in the cell viability and cell staining assays.

Determination of mitochondrial membrane potential by JC-1 staining:

JC-1 is a dye that can selectively enter into mitochondria and exhibits an intense red fluorescence in healthy mitochondria with normal membrane potentials. In cells with reduced MMP, the red fluorescence disappears. In the present study, Molt-3 leukemic cells were treated with different concentrations of the methanol extract of *Citrus limetta*, *Citrus sinensis* pulp and naringenin and the result of the flow cytometric analysis showed that there was a decrease in the mitochondrial membrane potential of the Molt-3 cells treated with citrus fruit pulp extracts and naringenin while much difference was not seen in normal peripheral blood lymphocytes. This indicates that the citrus fruit pulp extracts and naringenin were able to trigger intrinsic apoptotic pathway which might be due to the activation of Bcl-2 pathway by the attachment of Bax and Bad dimer to the outer mitochondrial membrane which lead to the loss of mitochondrial membrane potential.

Discussion:

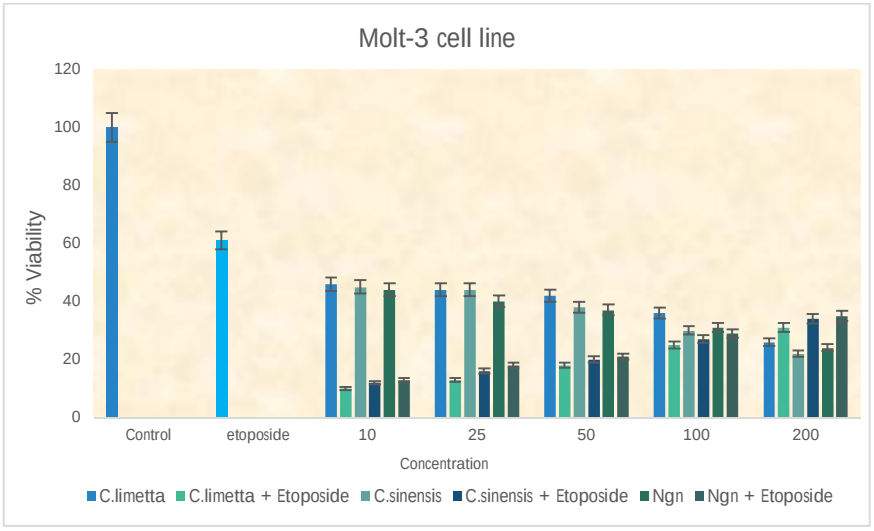
Apoptosis is a strictly controlled pathway which is responsible for removal of unwanted cells, old and injured cells. It is one of the major mechanisms of cell death in response to cancer therapies. Alterations in susceptibility to apoptosis not only contribute to neoplastic development but also can enhance resistance to conventional anticancer therapies, such as radiation and cytotoxic agents. Induction of apoptosis is one the most important marker of cytotoxic antitumor agents. Numerous studies have shown that some natural compounds induce apoptotic pathways that are blocked in cancer cells [17, 18]. Phytochemicals have shown promising results in cancer chemoprevention against various malignancies. Emerging evidences have shown the effects of plant-derived compounds on cell cycle regulatory and apoptotic pathways. Plant polyphenols are known to possess anticancer properties by interfering with the different stages of cancer development and also possess strong antioxidant activity which might be responsible for their cancer chemopreventive and therapeutic effects [19]. The results of various spectrophotometric and staining assays revealed that the citrus fruit pulp extracts and naringenin were able to induce cell death via apoptosis in Molt-3 cell line and also found to be not toxic to the normal peripheral blood lymphocytes. The results of the present study revealed that the methanol extract of *Citrus limetta* and *Citrus sinensis* pulp exhibited better cytotoxic activity when compared to naringenin which might be due to the synergistic effect rendered by various bioactive components present in the pulp extract including naringenin. To further confirm the mode of cell death Annexin V/FITC and JC-1 staining (flow cytometric analysis) were carried out. The results of Annexin V/FITC staining showed that *Citrus limetta* and *Citrus sinensis* fruit extracts along with naringenin were able to induce apoptosis at a later stage which eventually results in the loss of mitochondrial membrane potential. The loss of MMP was studied JC-1 staining. The results clearly showed that there was a great loss in mitochondrial membrane potential. Mitochondrial integrity is controlled by various members of the Bcl-2 superfamily such as Bax, Bid, Bak, Bad, Noxa, and PUMA as pro-apoptotic family members, while Bcl-xL, Bcl-2, Mcl-1, and A1 as antiapoptotic family members. During apoptosis, proapoptotic Bax and Bak undergo dimerization and insert into the outer mitochondrial membrane, triggering the intrinsic apoptotic pathway which results in the loss of mitochondrial

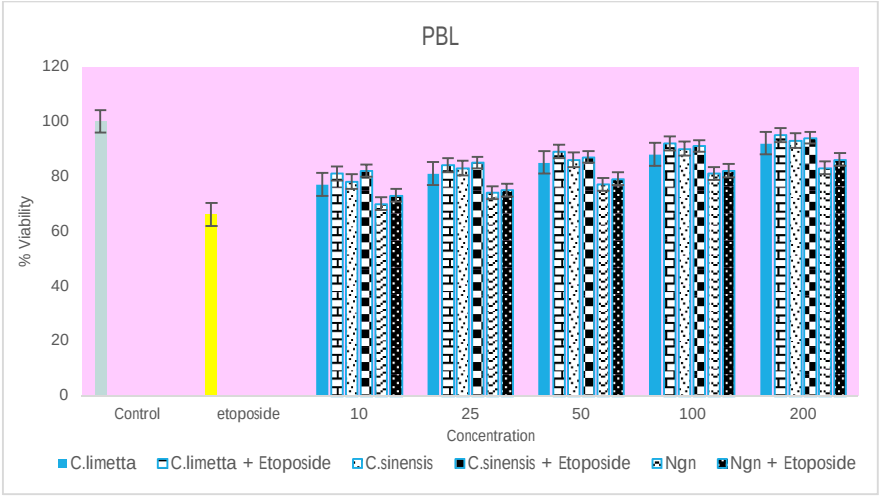
membrane potential [20]. Thus, from the above results it is clearly evident that methanol extract of *Citrus limetta* and *Citrus sinensis* pulp and naringenin were able to activate intrinsic apoptotic pathway in Molt-3 leukemic cells.

Conclusion:

To conclude, the methanol extract of *Citrus limetta* and *Citrus sinensis* pulp and naringenin were able to induce cell death in Molt-3 cells but were found to not toxic to the normal peripheral blood lymphocytes. The results of flow cytometric analysis confirmed that the extracts trigger intrinsic apoptotic pathway in Molt-3 cell line. The anticancer potential of citrus fruit pulp extracts might be due to the presence of bioactive constituents including naringenin, a flavonone. Eventhough, further *in vitro* and *in vivo* investigations has to be carried out for the better understanding of the mode of action of citrus fruit extracts and naringenin.

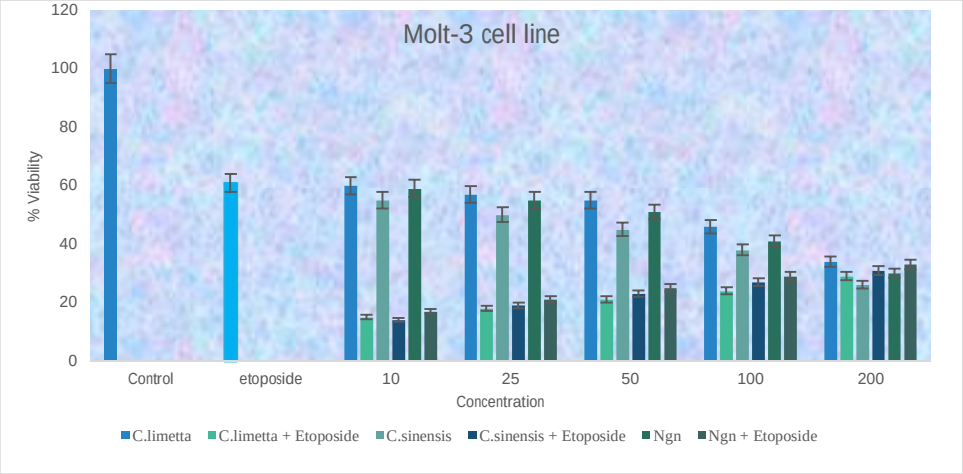
Figure 1: Cytotoxic effect of methanol extract of *Citrus limetta, Citrus sinensis* pulp and naringenin as determined by MTT assay

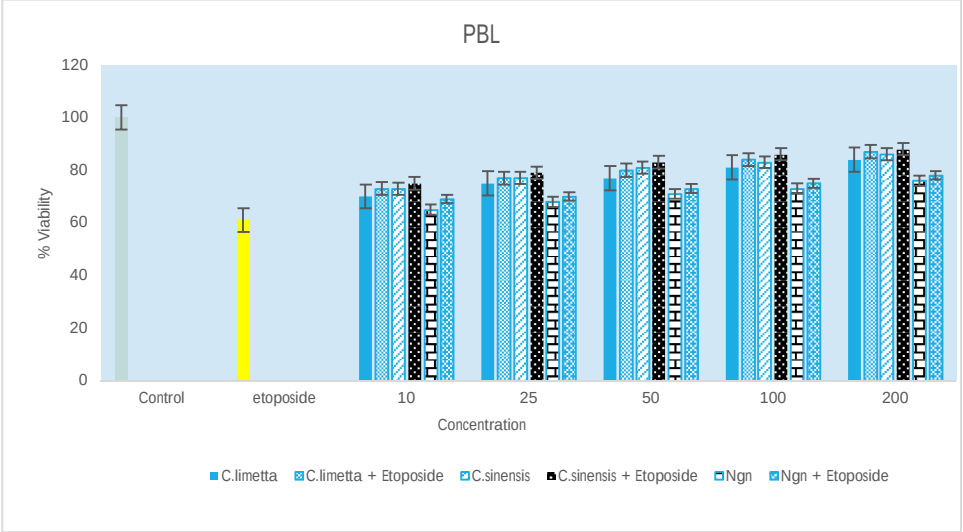




Concentration of Citrus fruit - µg/ml and Ngn- µM

Figure 2: Cytotoxic effect of methanol extract of *Citrus limetta, Citrus sinensis* pulp and naringenin as determined by SRB assay





Concentration of Citrus fruit - µg/ml and Ngn- µM

Table 1: Effect of *Citrus limetta* and *Citrus sinensis* pulp extracts on morphological and nuclear changes in Molt -3 cells (Giemsa and Ao/EtBr staining)

Concentration (50µg/ml)	No. of apoptotic cells/100 cells						Apoptotic ratio					
	Without Etoposide			With Etoposide			Without Etoposide			With Etoposide		
	CL	CS	Ngn	CL	CS	Ngn	CL	CS	Ngn	CL	CS	Ngn
Giemsa	57	61	57	80	82	79	1.3	1.5	1.32	4.56	4.0	3.7
Ao/EtBr	58	63	56	79	83	75	1.70	1.7	1.27	3.76	4.9	3.0

Values are mean of triplicates

Plate 1: Giemsa and Ao/EtBr staining in Molt-3 cells

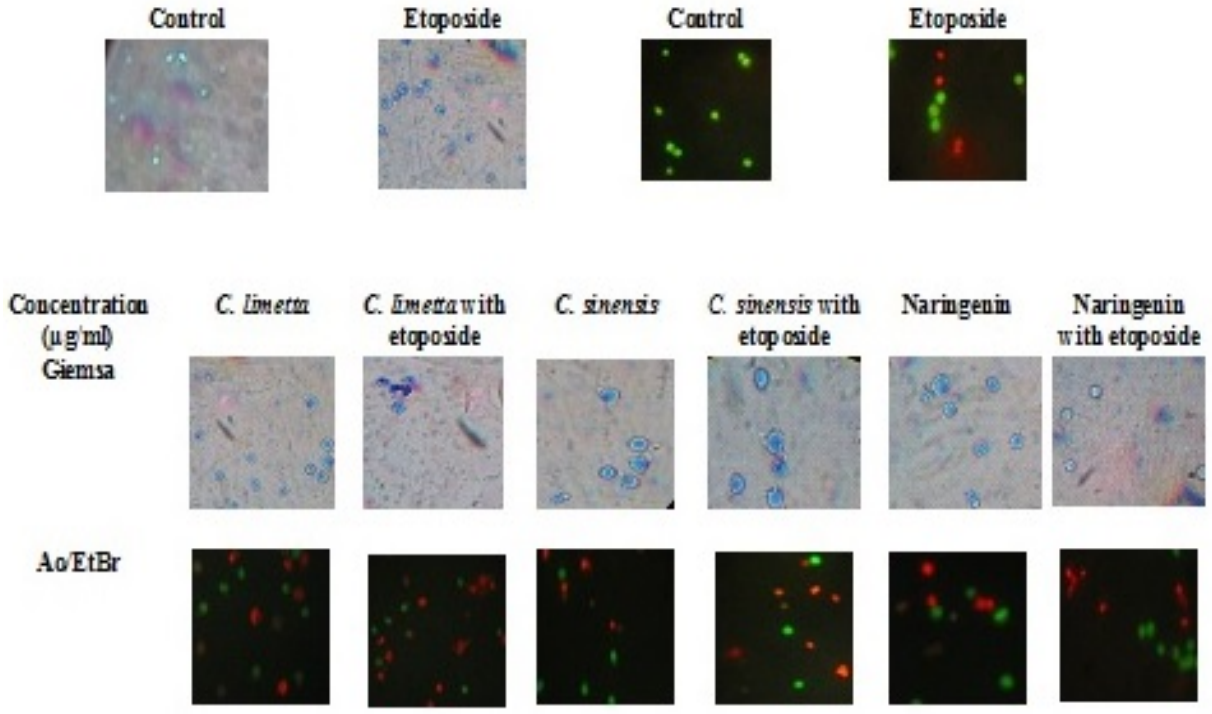


Table 2: Effect of *Citrus limetta* and *Citrus sinensis* pulp on morphological and nuclear changes in PBL (Giemsa and AO/EtBr staining)

Concentration (50µg/ml)	No. of apoptotic cells/100 cells						Apoptotic ratio					
	Without Etoposide			With Etoposide			Without Etoposide			With Etoposide		
	CL	CS	Ngn	CL	CS	Ngn	CL	CS	Ngn	CL	CS	Ngn
Giemsa	15	15	23	11	11	21	0.17	0.17	0.29	0.12	0.12	0.26
Ao/EtBr	23	19	24	20	17	22	0.29	0.23	0.31	0.25	0.20	0.28

Values are mean of triplicates

Plate 2: Giemsa and AO/EtBr staining in PBL

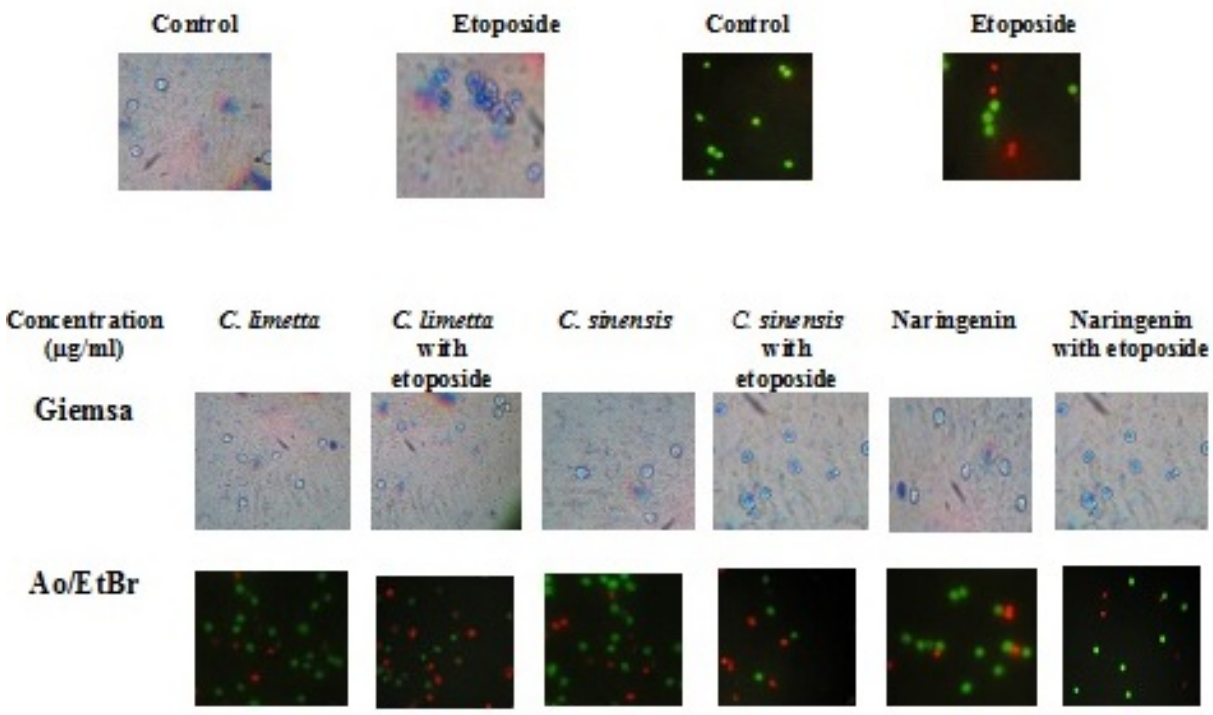


Figure No 3A: Annexin V-/FITC staining (Molt-3 cells)

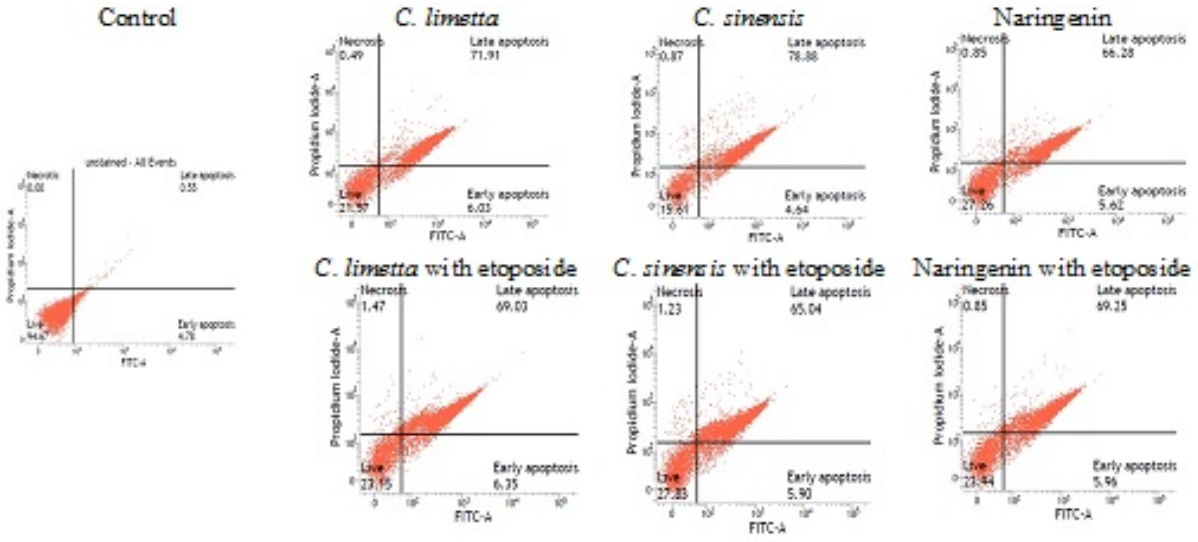


Figure No 3B: Annexin V-/FITC staining (PBL)

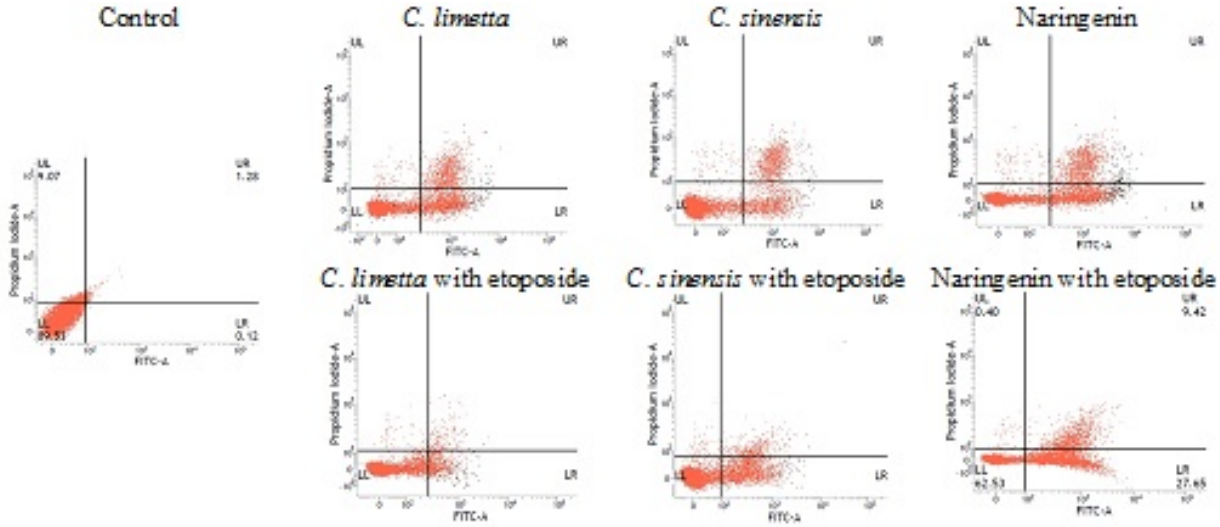


Figure 4A: JC-1 staining (Molt-3 cells)

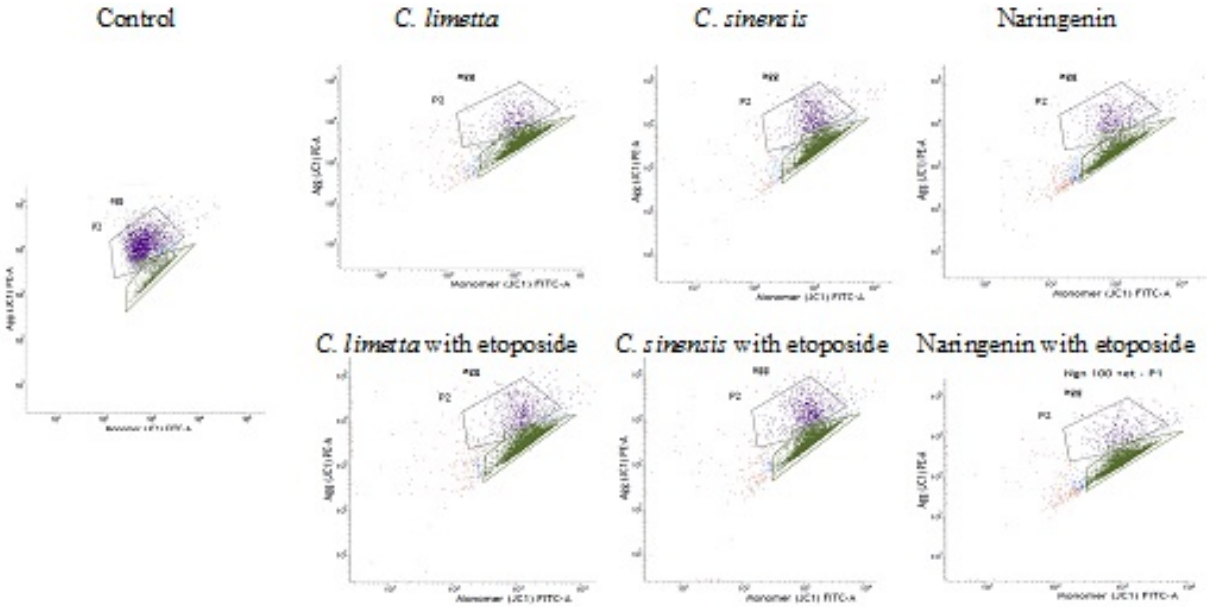
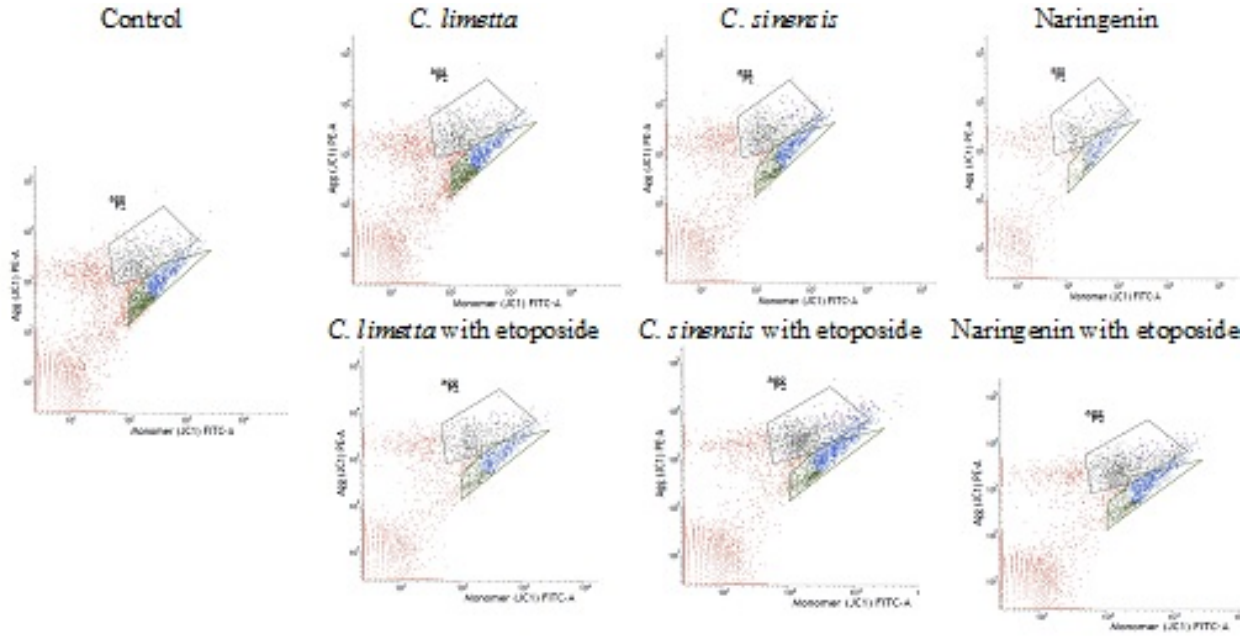


Figure No 4B: JC-1staining (PBL)



References:

1. Miladinia M, Baraz S, Javadi M, Nouri EM and Baeis MG. Study Gaps Relevant to Use of Complementary Medicine in Patients With Leukemia: A Review Study. Jundishapur J Chronic Dis Care 2016;5(3):e34033.
2. Luan C, Yang Z, Chen B. The functional role of microRNA in acute lymphoblastic leukemia: relevance for diagnosis, differential diagnosis, prognosis, and therapy. OncoTargets and Therapy 2015;8:2903–14.
3. Babu KLG, Mathew J, Doddamani GM, Narasimhaiah JK, Naik LR. Oral health of children with acute lymphoblastic leukemia. A review J Orofac Sci 2016;8:3-11.
4. Chinembiri TN, Du Plessis LH, Gerber M, Hamman JH and Du Plessis J. Review of Natural Compounds for Potential Skin Cancer Treatment. Molecules 2014;19:11679-721.
5. Pavithra K, Vadivukkarasi S. Evaluation of free radical scavenging activity of various extracts of leaves from *Kedrostis foetidissima* (Jacq.). Food Sci Human Wellness 2015;4:42–6.
6. Chaudhari SN, Pathan AH. Study of development of Promo-lime nectar fortified with sweet lime peel powder. Int J Sci Res 2014;4(10):836-39.
7. Mushtaq M, Sultana B, Bhatti HN and Asgher M. Optimization of enzymes-assisted revalorization of sweet lime (*Citrus limetta*) peel into phenolic antioxidants. Bio Resources 2014;9(4):6153-65.
8. Yen HR, Liu CJ, Yeh CC. Naringenin suppresses TPA-induced tumor invasion by suppressing multiple signal transduction pathways in human hepatocellular carcinoma cells. Chemico-Biological Interactions 2015;235:1–9.
9. Montecucco A, Zanetta,Z, Biamonti G. Molecular mechanisms of etoposide. EXCLI Journal 2015;14:95-108
10. Igarashi M, Miazawa T. The growth inhibitory effect of conjugated linoleic acid on a hepatoma cell line Hep G2, is induced by a change in fatty acid in cells. Biochem et Biophysica Acta/Mol Cell Bio Lipids 2001;1530:162-71.
11. Skehan P, Storeng R, Scudiero D, Monks A, McMahon J, Vistica D, *et al*. New colorimetric cytotoxicity assay for anticancer drug screening. J Natl Cancer Inst 1990; 82:1107-12.
12. Chin HW, Chill HF, Tang KS, Chang FR, Wu YC. Bullatacin a potent antitumour annonaceou sacetogenin, inhibits proliferation of human by apoptosis induction. Life Sci 2001;69:1321-31.
13. Parks DR, Bryan VM, Oi VT, Herzenberg LA. Antigen-specific identification and cloning of hybridomas with fluorescence-activated cell sorter. Proc Nat Acad Sci USA 1979;76:1962-66.
14. Rosangkima G, Jagetia GC. Cytotoxic potentials of alkaloid extract of *solanum khasianum* berries against dalton's lymphoma, MCF-7 and HELA cell lines *in vitro*. World J Pharm Pharma Sci 2015;4:891-904.
15. Henry CM, Hollville E, Martin SJ. Measuring apoptosis by microscopy and flow cytometry. Methods 2013;61:90–7.
16. Thani NAA, Keshavarz S, Lwaleed BA, Cooper AJ, Rooprai HK. Cytotoxicity of gemcitabine enhanced by polyphenolics from *Aronia melanocarpa* in pancreatic cancer cell line AsPC-1. J Clin Pathol 2014;67:949–54.
17. Safarzadeh E, Shotorbani SS, Baradaran B. Herbal Medicine as Inducers of Apoptosis in Cancer Treatment. Adv Pharm Bull 2014;4:421-27.
18. Kang MH, Reynolds CP. Bcl-2 Inhibitors: Targeting Mitochondrial Apoptotic Pathways in Cancer Therapy. Clinical Cancer Research 2009:15:1126–32.
19. Gali-Muhtasib H, Raed Hmadi HR, Kareh M, Tohme R and Darwiche N. Cell death mechanisms of plant-derived anticancer drugs: beyond apoptosis. Apoptosis 2015; DOI 10.1007/s10495-015-1169-2.
20. Mukhopadhyay S, Panda PK, Sinha N, Das DN, Bhutia SK. Autophagy and apoptosis: where do they meet? Apoptosis 2014;DOI 10.1007/s10495-014-0967-2.