Research Article

Cytotoxic Effect of *Citrus limetta* and *Citrus sinensis* on Molt-3 Cell Line

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ABSTRACT

The cytotoxic effect of the methanol extract of *Citrus limetta* and *Citrus sinensis* pulp was assessed by MTT and SRB assay in both normal/untransformed cells (*Saccharomyces cerevisiae*) and leukemic cells (Molt-3) in the present study. The experiment was performed using varying concentrations ranging from 50, 100, 200, 400, 500 and 1000µg of the methanol extract of *Citrus limetta* and *Citrus sinensis* pulp. Both the pulp extracts were able to protect *Saccharomyces cerevisiae* from oxidant induced damage caused by H_2O_2in a dose dependent manner. In Molt-3 leukemic cell line, the results of MTT assay showed that fruit pulp extracts induced 50% cell death at a lower concentration of 50µgwithin 24 hours of treatment. Similarly, in SRB assay, both the extracts were able to cause cell death in a dose dependent manner. To conclude, the methanol extract of *Citrus limetta* and *Citrus sinensis* pulp exhibited significant anticancer activity towards Molt-3 cell line while rendering protection to untransformed *S. cerevisiae* cells from oxidative damage.

Keywords: Anticancer, Molt3 cell line, *S. cerevisiae*, *Citrus* fruits.

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]. Leukemia is the most common childhood cancer in India with the relative proportion varying between 25% and 40%. Among various leukemias, acute lymphoblastic leukemia (ALL) accounts for 60-85% of all cases in children in India and its incidence peaks from 2 to 5 years of age, while it is relatively rare in adults [2, 3]. Due to the limited success of clinical therapies including radiation, chemotherapy, immunomodulation and surgery in treatment of cancer, there is an imperative need of alternative strategies in cancer management [4]. Therefore, efforts are made to search for effective naturally occurring anticarcinogens that would prevent, slow, or reverse cancer development. Medicinal plants have a remarkable role in the management of cancer. It is estimated that plant-derived compounds constitute more than 50% of anticancer agents. Numerous cancer research studies have been conducted using traditional medicinal plants in an effort to discover new therapeutic agents that lack the toxic side effects [5].

The *Citrus* species are the important source of valuable oil which might be utilized for edible and other industrial applications and essential oils are broadly used as pharmaceutical components, in nutritious supplements and for cosmetic industry and armotherapy [6]. *Citrus limetta* is commonly known as Mousambi or sweet lime in India. This has been used for several medicinal purposes. *C. limetta* is known to be an antihyperglycemic plant, its fruit and leaves are used for common cold, decreasing cholesterol level, fever regulation, regulating inflammation, digestive disorder and as well as blood pressure modulator [7,8]. *Citrus sinensis* is an important nutritional source for human health and it has immense economic value. *Citrus sinensis* is a powerful natural antioxidants, an excellent source of vitamin C that builds the body's immune system. Biologically active compounds like liminoids, synephrine, polyphenols, pectin and sufficient amount of potassium, thiamine, folacin, calcium, niacin and magnesium are useful to prevent cancer, atherosclerosis, kidney stonesand stomach ulcers. Traditionally these Citrus fruits occupies a significant place in therapeutic aspects such as anti-cancer, anti-inflammatory, antitumour and blood clot inhibition activities and also based on the availability of these fruit varieties [9], *C. limetta* and *C. sinensis* were chosen for the present study. Thus, the present study aimed to investigate the antileukemic property of the methanol extract of *C. limetta* and *C. sinensis* pulp.

MATERIALS

MTT (3-(4,5- dimethylthiozol-2-yl)-2,5-diphenyltetrazolium bromide), RPMI 1640, PBS (Phosphate buffered saline), Isopropanol, Sulphorhodamine B (SRB), Acetic acid, Tris, Tricholoroacetic acid, Hydrogen peroxide and Etoposide.

METHODS

Sample collection and preparation

Fresh fruits (*Citrus limetta* and *Citrus sinensis*)were procured from local markets in Coimbatore, India. The

peels and pulp were separated and sliced into small pieces. The pulp was kept in an incubator for 12 hours at 40℃. About 20g of pulp is weighed and mixed with 100ml of methanol andkept overnight in shaker incubator. Then the extracts were filtered and used for the analysis performed in the study.

Maintenance of Molt-3 cell line and *Saccharomyces cerevisiae*

Molt-3 T-cell acute lymphoblastic leukemic cell line, was purchased from NCCS, Pune, India. It was cultured using RPMI 1640 medium supplemented with 10% FBS and 1mM sodium pyruvate and incubated at 37˚C. The cells (1×10^6) were seeded onto 96 well plate for cell viability assays.

Saccharomyces cerevisiae cells were inoculated and incubated at 37˚C for overnight. The cells were harvested and were tested with various concentrations ranging from 50, 100, 200, 400, 500 and 1000µg of methanol extract of *Citrus limetta* and *Citrus sinensis* pulp. Oxidative stress was induced using H_2O_2 at a concentration of 200 µM. Cytotoxicity was determined by MTT/SRB assay.

MTT dye reduction assay

MTT assay was carried out by following Igarashi and Miyazawa, 2001 protocol[10].The treated Molt-3 cells and *S. cerevisiae* were incubated with 50µl of MTT at 37°C 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). The optical density of the control cells were fixed to be 100% viable and the percent viability of the cells in the treatment groups were calculated using

Percent cell viability = [(C− T)] / (C)] ×100

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

SRB assay

SRB assay was done using the method adopted by Skehan *et al*., 1990[11]. The treated Molt-3 cells and *S. cerevisiae* 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 4°C for one hour after which they were washed 5 times with 200µl of ice cold PBS. The PBS was removed and SRB (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 pellets were 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 microtiter plate and read at 490nm. The cell survival was measured as the percentage absorbance compared to the control (untreated cells). The percent viability of the cells in the treatment groups were calculated using

Percent cell viability = [(C− T)] / (C)] ×100

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

RESULTS AND DISSCUSSION

In order to determine the cytotoxic effect of the methanol extract of *Citrus limetta* and *Citrus sinensis* pulp, MTT and SRB assays were performed in both normal/ untransformed cells (*Saccharomyces cerevisiae*) and leukemic cells (Molt-3). The experiment was performed using varying concentrations ranging from 50, 100, 200, 400, 500 and 1000µg. The results revealed that, upon treatment with H_2O_2, there was a steep decrease in the cell survival rate and addition of the methanolic extract of both the fruit pulp showed an improvement in the viability of*S. Cerevesiae* cells indicating its protective property in untransformed cells as indicated in the Figure 1 and 2. Both the fruit pulp extracts exhibited a similar activity in a dose dependent manner. As the concentration increases, the cell viability also increased in both the cytotoxic assays performed.

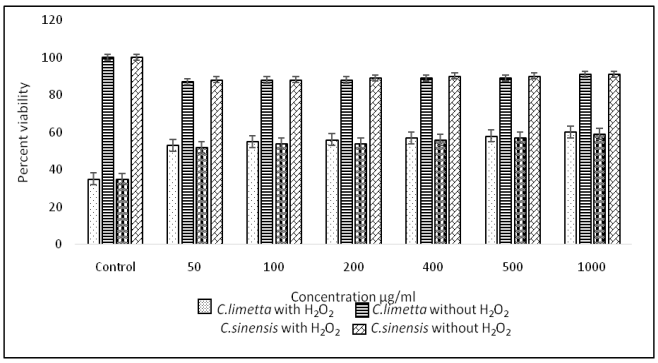

Figure 1: Cytotoxic effect of methanol extract of *Citrus limetta* and *Citrus sinensis* pulp extracts in *S. cerevisiae* as determined by MTT assay.

Dwivedi *et al*., (2013) evaluated that the ethanol and chloroform extracts of *Cissus quadrangularis* exhibited more cytotoxicity towards HeLa cell line and less toxicity towards normal monkey kidney cell line VERO as estimated by MTT assay[12].Harput *et al*, (2012) investigated the antioxidant and cytotoxic effects of *Moltkia aurea Boiss.* In this study, three cancerous and one non-cancerous cell lines were used for MTT assay. The results revealed that aqueous extract of *Moltkia aurea* showed more cytotoxicity to the three different cancer cell lines such as Hep-2, RD and L-20B, but it did not show any cytotoxicity towards the non-cancer cell line (VERO)[13].

Hydrogen peroxide is a biologically relevant, non-radical reactive oxygen species and is inevitably generated as a by-product of normal aerobic metabolism. However, when concentration increases under stress conditions, H_2O_2 could be detrimental for cells and, furthermore, could be converted into other ROS such as hydroxyl radicals.

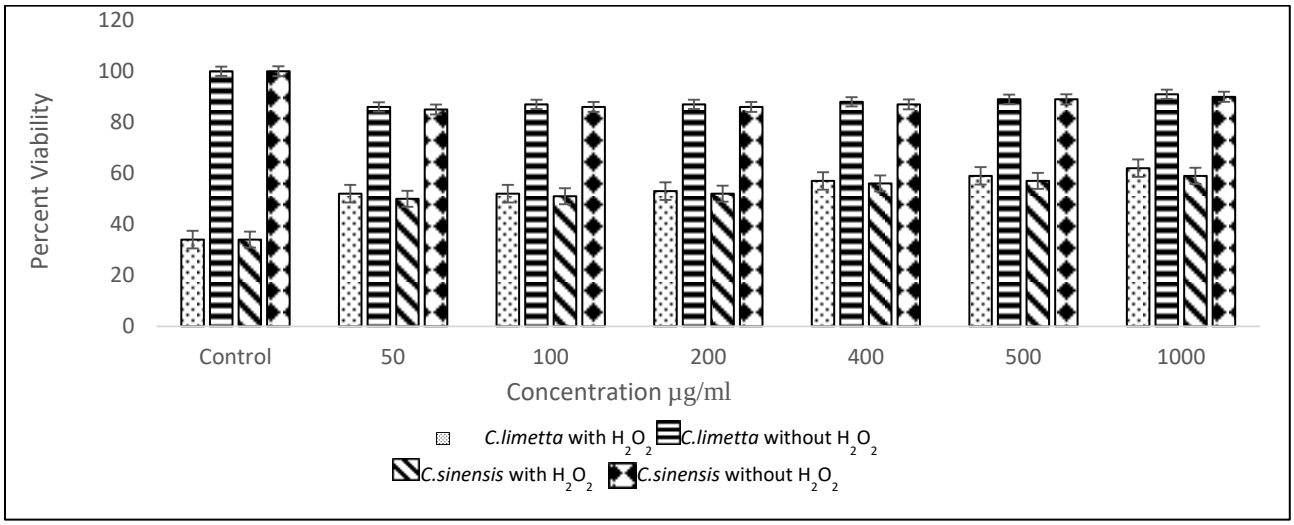

Figure 2: Cytotoxic effect of methanol extract of *Citrus limetta* and *Citrus sinensis* pulp extracts in *S. cerevisiae* as determined by SRB assay

Thus, elimination or neutralization of H_2O_2 is crucial for the cells[14].In the present study, upon treatment with H_2O_2, the viability of yeast cells were decreased but addition of the Citrus fruit pulp extracts increasedthe viability thereby rendering protective effect against H_2O_2 induced toxicity in yeast cells.

Dose optimization of the extracts in Molt-3 leukemic cells

To determine the optimal concentration of the methanol extract of *C. Limetta* and *C. Sinensis* fruit pulp at which the maximum efficacy of the extract observed,, MTT assay was performed using various concentrations of pulp extract at different time intervals 6,12,24,36, 48 and 72 hours in Molt-3 cell line (leukemic cell line).The results are presented in Figure 3 and 4. It is evident from the graph that when the pulp extract concentration is lower, cytotoxicity was observed till 12 hours of exposure beyond which, no significant cytotoxicity was observed. In addition, increase in cell viability was observed after 12 hours of exposure. While at higher concentration of the pulp extract, decreased cell viability was recorded till 36 hours of treatment. Hence, the optimal dose was found to be 50µg/ml.

Tajudin *et al*., investigated the cytotoxic effect of *Cynometra cauliflora* whole fruit extract towards HL-60(Human promyelocytic leukemic cell line) and NIH/3T3 cells by measuring the cell viability using MTT assay with different concentrations of extract. The results showed that the methanol extract was able to induce apoptosis in concentration dependent manner[15].The results of present study indicate that the fruit pulp extract could cause cytotoxicity to Molt-3 leukemic cellsonly for short time when it is administered at a lower concentration. However, the cytotoxic action is prolonged to 36 hours when a higher concentration of the pulp extract is supplemented. Also, it was evident that maximum inhibition rendered by both Citrus species was at 24 hour. Hence the optimal time for treatment of the cells was fixed as 24 hour.

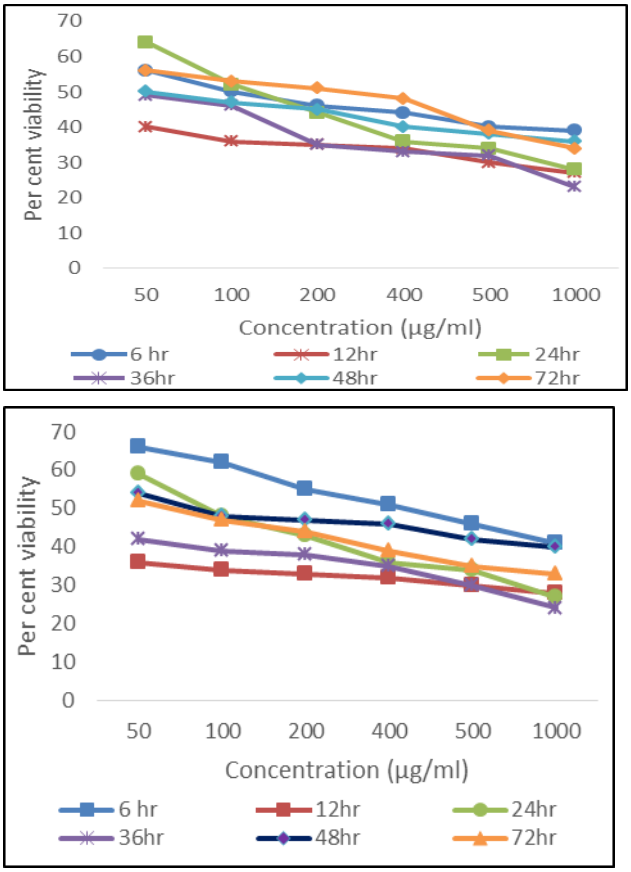

Figure 3 & 4: Dose optimization in Molt-3 cell line *Citrus limettaCitrus sinensis*

SRB assay

To confirm the cytotoxic effect of the methanol extract of *Citrus limetta* and*Citrus sinensis* pulp, SRB assay was performed along with the standard chemotherapeutic drug etoposide at a concentration of 200µM.The results revealed that the methanol extract of both the fruits caused cell death in the leukemic cells when compared

to the standard etoposide. In the present study, the methanol extract of both fruits pulp exhibited cytotoxicity towards leukemic cell line even at a lower concentration of 50µg/ml and also increase in cell death was observed with increase in concentration which is depicted in Figure 5 and 6.

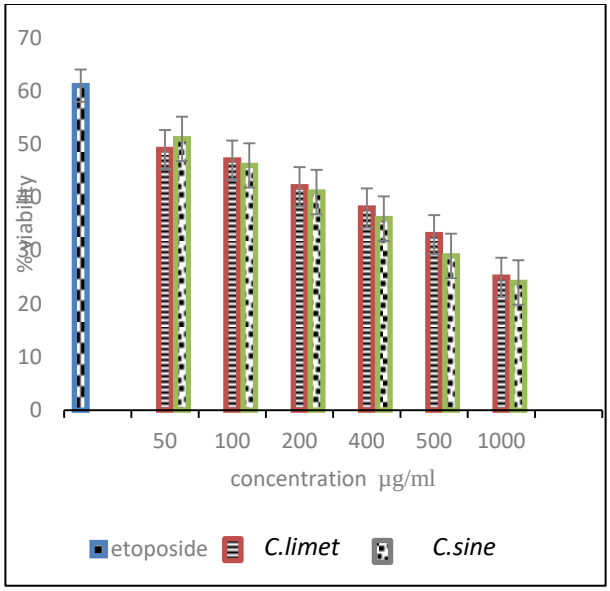

Figure 5: Effect of methanol extract of *C. limetta and C. sinensis* pulp on Molt-3 leukemic cell line as determined by SRB assay

In vitro anti proliferative activity by Dzoyem *et al.* (2013) revealed that, extract from the bark of *F. heitzii* and extract from *H. lyrata* roots had significant cytotoxic activity on THP-1 (leukemic) and PC-3 (prostrate) cell line respectively[16]. All the non-polar extracts (hexane, benzene, chloroform, ethyl acetate) of S. xanthocarpum (Sx) fruits exhibited greater anticancer potential against THP-1 (leukemic cell line) while benzene, chloroform fractions showed better cytotoxicity against lung cancer cell line (HOP-62) [17]. In accordance with these findings, the current investigation revealed the Citrus fruit pulp extracts which were able to protect the normal cells (*S. cerevisiae*) from induced oxidative damage simultaneously enhancing cell death in leukemic cells in a dose dependent manner thereby indicating that the pulp extracts could cause cytotoxicity only in leukemic cells and hence the fruit pulp extract can readily be used for therapeutic purposes and can be used in the preparation of drugs for leukemia.

CONCLUSION

To conclude from the above results, the methanol extract of *Citrus limetta* and *Citrus sinensis* pulp showed the cytotoxicityin comparison with standard chemotherapeutic drug etoposide in Molt-3 leukemic cell lines. The optimal dose and time period for exerting the activity was found to be 50µg at 24 hour interval. And also both the extracts mediated protection against oxidant (H_2O_2) induced damage to the untransformed *S. cerevisiae* cells. It is evident that cell death occurs on treatment with the *Citrus* fruit extracts in leukemic cell line but whether the cell death is mediated by apoptosis or necrosis is not clear.Hence, further studies need to be conducted, in order to understand the actual mechanism by which the *Citrus* fruits rendered protection to normal cells while causing damage to the Molt-3 leukemic cells.

REFERENCES

1. Zhou J, Sam Wang S, Sun K and Chng W,The emerging roles of exosomes in leukemogeneis, Oncotarget, 2016,1-10.
2. Luan C, Yang Z and Chen B, The functional role of microRNA in acute lymphoblastic leukemia: relevance for diagnosis, differential diagnosis, prognosis, and therapy, OncoTargets and Therapy, 8, 2015, 2903–2914.
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. Tagne RS, Telefo BP, Nyemb JN, Yemele DM, Njina SN, Goka SMC, Lienou LL, Kamdje AHN, Moundipa PF and Farooq AD,Anticancer and antioxidant activities of methanol extracts and fractions of some Cameroonian medicinal plants, Asian Pac J Trop Med, 7, 2014, S442-S447.
5. Shaikh R, Pund M, Dawane A and Iliyas S, Evaluation of Anticancer, Antioxidant, and Possible Anti‐inflammatory Properties of Selected Medicinal Plants Used in Indian Traditional Medication, Journal of Traditional and Complementary Medicine, 4, 2014, 253‐257.
6. Juárez MCC, Núñez RER, Álvarez JEB, González GMM, Bolaños JLN, Islas HJ, Characterization of volatile compounds in the essential oil of sweet lime (*Citrus limetta* risso), Hilean JAgri Res, 72(2), 2012, 275 – 280.
7. KunduSen S, Haldar PK, Gupta M, Mazumder UK, Saha P, Bala A, Bhattacharya S, Kar B, Evaluation of antihyperglycemic activity of *Citrus limetta* fruit peel in streptozotocin-induced diabetic rats, ISRN Endocrinology, 2011, 1-6.
8. Gill NS and Katoch M, An updated review of *Citrus limetta* Risso, The Global J PharmaRes, 3(1), 2014, 1910-20.
9. Etebu E and Nwauzoma AB, A Review On Sweet Orange (*Citrus Sinensis* L Osbeck): Health, Diseases and Management, Am J Res Communication, 2(2), 2014, 33-70.
10. Igarashi Mand 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. Biophys. Acta/ Molecular and Cell biology of lipids, 1530, 2001, 162-171.
11. Skehan P, StorengR, Scudiero D, Monks A, McMahon J, Vistica, D, New colorimetric cytotoxicity assay for anticancer drug screening, J. Natl. Cancer. Inst., 82, 1990, 1107-1112.
12. Dwivedi A, Seethalakshmi I and Sharmila D, Anticancer properties of *Cissus quandrangularis*, J Chem Pharm Res 5, 2013, 135-139.

13. Harput US, Nagatsu A and Saracoglu I, Antioxidant and cytotoxic effect of *Moltkia aurea* Boies, Rec. Nat. Prod, 6, 2012, 62-66.

14. Gul MZ, Ahmad F, Kondapi AK, Qureshi IA and Ghazi IA, Antioxidant and antiproliferative activities of *Abrus precatorius* leaf extracts, BMC Complementary and Alternative Medicine, 13, 2013, 1-12.

15. Tajudin TJSA, Mat N, Aishah ABS,Yusran AAM, Alwi A and Ali AM, Cytotoxicity, Antiproliferative Effects, and Apoptosis Induction of Methanolic Extract of *Cynometra cauliflora* Linn. Whole Fruit on Human Promyelocytic Leukemia HL-60 Cells, Evidence-Based Complementary and Alternative Medicine, doi:10.1155/2012/127373.

16. Dzoyem JP, Guru SK, Pieme CA, Kuete V, Sharma A, Khan IA, Saxena AK and Vishwakarma RA, Cytotoxic and antimicrobial activity of selected Cameroonian edible plants, BMC Compl. Alter. Med., 13, 2013, 1-6.

17. Chaudary A, Rampal G, Sharma U, Thind TS, Singh B, Vig AP and Arora S, Anticancer, antioxidant activities and GC-MS analysis of glucosinolates in two cultivars of broccoli, Med. Chem. Drug Discov, 2, 2013, 30-37.

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