



Original article

D-limonene possesses cytotoxicity to tumor cells but not to hepatocytes

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ABSTRACT

Introduction: K562, human chronic myeloid leukemia cell line shows presence of constitutively active BCR-ABL gene. D-limonene, monoterpenes obtained from essential oils of citrus fruits have exhibited its antitumor activity in various types of cancers. Murine xenograft models are used for estimation of in vivo effect of D-limonene in K562 tumor xenograft model.

Aim: The aim of present study was to evaluate the in vitro and in vivo effect of D-limonene on primary hepatocytes and K562 tumor implanted C57BL/6 mice respectively.

Material and methods: Effect of D-limonene on growth of K562 cells and mouse primary hepatocytes was determined in vitro by MTT assay. The in vivo effect of D-limonene was also determined on chemically immunocompromised K562 tumor xenografted C57BL/6 mice.

Results and discussion: In vitro dose dependent and time dependent studies of D-limonene shows significant reduction in viability of K562 cells. Dose dependent studies of doxorubicin and D-limonene treatment for 48 h shows significant reduction in viability of primary hepatocytes with doxorubicin whereas, the reduction was non significant with D-limonene. D-limonene treatment for 14 days also shows dose dependent reduction in tumor volume in K562 tumor xenograft C57BL/6 mice.

Conclusions: D-limonene inhibited the growth of primary hepatocytes in-vitro and also inhibited in vivo K562 tumor growth in C57BL/6 mice, which suggests safety and efficacy of D-limonene in the treatment of chronic myeloid leukemia.

1. INTRODUCTION

Chronic myeloid leukemia (CML) is a hematopoietic stem cell disorder characterized by the presence of the Philadelphia (Ph) chromosome, resulting from translocation of *abl* proto-oncogene on chromosome 9 and the *bcr* gene on chromosome 22, t(9;22)(q34;q11) and generation of a chimeric BCR-ABL gene, a constitutively active tyrosine kinase that expresses P210BCR-ABL fusion protein of 210kDa.^{1,2} Imatinib and other tyrosine kinase inhibitors (TKIs) are used as first-line treatment of CML, has increased the survival rate in patients with fewer side effects as compared with treatments such as interferon and hydroxyurea.³⁻⁵ Imatinib and other TKIs eventually seems to stop working caused by changes in the genes of the CML cells which known as resistance.⁶⁻⁸ However, the emergence of resistance to first-line therapy in CML patients leads to search for new molecule for the treatment of the disease.^{9,10}

Monoterpenes, non-nutritive dietary components found in the essential oils of citrus fruits and other plants have exhibited its antitumor activity in various types of cancers.^{11,12} D-limonene ((R)-1-methyl-4-(1-methylethenyl) cyclohexene), a monocyclic monoterpene abundantly found in volatile oils of citrus fruits such as orange, lemon, grapefruit and others plants has inhibited neoplasia in animal models and cell growth in-vitro.¹³⁻¹⁵ Oral administration of D-limonene inhibited the growth of rodent pancreatic, mammary and gastric carcinogenesis and exhibited anticancer activity.¹⁶⁻²⁰

2. AIM

The aim of present study is to evaluate the in vitro effect of D-limonene on primary hepatocytes and in vivo effect on K562 cells implanted C57BL/6 xenograft mice.

3. MATERIAL AND METHODS

3.1. Material

D-limonene was purchased from MP Biomedicals Solon, OH, USA. Cell culture medium Roswell Park Memorial Institute medium (RPMI)-1640, Dulbecco's modified Eagle medium (DMEM), trypsin, fetal bovine serum (FBS), HEPES, MTT, collagenase, sodium bicarbonate and trypan blue were obtained from Himedia, Mumbai, India. Antibiotic-antimycotic solution was obtained from Gibco, Grand Island, NY, USA. Doxorubicin was purchased from Sigma-Aldrich, St Louis, USA. Cyclosporine (Sandimmune) from Novartis, Basel, Switzerland and Ketoconazole (Nizral) from Johnson&Johnson, New Brunswick, New Jersey, USA. Cyclophosphamide (Endoxan) was purchased from Baxter, Halle, Germany. Ampoxin was purchased from Unichem laboratories Ltd., Mumbai, India. Rodent diet was obtained from VRK Nutrition, Pune, India.

3.2. Cell and cell culture

Human CML cell line K562 was purchased from National Centre for Cell Science (Pune, India, NCCS). Cells were maintained in RPMI-1640 medium supplemented with 10% FBS with 1% antibiotic-antimycotic solution. Cells were maintained in an incubator at 37°C supplemented with 5% CO₂ atmosphere. Cells used in the study were in the logarithmic growth phase.

3.3. Experimental animals and housing conditions

Healthy 4-6-week-old C57BL/6 male mice weighing 25-30 g were purchased from Mahaveera Enterprises, Hyderabad, India. Mice were housed under standard laboratory conditions (with a relative humidity of 60% ± 5% and a temperature of 25°C ± 2°C and 12 h : 12 h light and dark cycle) in individually ventilated cage. The mice were fed with autoclaved balanced rodent food pellet and Ampoxin (0.1 µg/mL) by drinking water was provided ad libitum. Mice were cared for and used in accordance with the CPCSEA guidelines and all animal experimental procedures were reviewed and approved by the Institutional Animal Ethics Committee (PERD/IAEC/2016/001) prior to initiation of the experiment.

3.4. Cell viability (MTT) assay of K562 cells

Effect of D-limonene on the viability of K562 cells was evaluated in time and dose dependent manner using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) conversion assay. K562 cells (104 cells/well) were seeded in 96 well plate containing RPMI 1640+10% FBS in presence of different concentrations of D-limonene. The cells were treated with D-limonene at concentration of 1 mM, 2 mM, 4 mM and 8 mM and doxorubicin as a standard at concentration of 0.4 µM, 0.8 µM, 1.6 µM and 3.2 µM for 24 h and 48 h. After the treatment, 20 µl of MTT (5 mg/mL) was added to each well and the cells were incubated at 37°C for another 4 h. The amount of 100 µL of dimethylsulfoxide (DMSO) was added in each well followed by removal of culture medium by centrifugation (200 g for 7 minutes). Then, the optical density (OD) was measured at 570 nm by a microplate reader (EL800 BioTek Instruments, Inc.) Experiments were carried out in quadruplicate.

3.5. Primary culture preparation of mouse hepatocytes

Mouse was anaesthetized and abdomen was opened to cannulate the portal vein with perfusion buffer.²¹ Gradually the perfusate flow was increased to wash out the blood from the liver (up to 20 mL/min) making an incision in vena cava to allowing perfusate efflux. The liver was blanched and was dissected out; perfusion was continued with warm collagenase (type II) buffer (0.05%). The liver was transferred to the petri dish containing ice-cold suspension buffer containing calcium and magnesium). Gilsson's capsule was dissected and suspension was filtered through cell strainer. Debris were discarded and washed with wash buffer (perfusion buffer containing calcium) by centrifugation (50 g for

5 minutes) and resuspension.^{22,23} Cell viability was determined using trypan blue method and cells were cultured in DMEM containing 10% FBS and maintained in an incubator at 37 °C supplemented with 5% CO₂ atmosphere.

3.6. Cell viability (MTT) assay on primary mouse hepatocytes

The primary hepatocytes of mouse were seeded in 96 well plate containing DMEM+10% FBS. Treatment with doxorubicin and D-limonene was given at different concentration after 24 h of plating. The cells were treated with doxorubicin at concentration of 2 μM, 4 μM, 8 μM, 16 μM and 32 μM and D-limonene with concentration of 2 mM, 4 mM, 8 mM, 16 mM and 32 mM for 48 h. The cytotoxicity of D-limonene and doxorubicin on primary hepatocytes was compared by addition of MTT as described above and absorbance was measured. Experiments were carried out in quadruplicates.

3.7. Tumor xenograft model

Healthy male C57BL/6 mice were immunocompromised by oral administration of ketoconazole 10 mg/kg and intraperitoneal cyclosporine 30 mg/kg every day for 7 days. Cyclophosphamide was given at a dose of 60 mg/kg subcutaneously on days 3 and 1 before tumor cell injection in all groups of animals. K562 cell suspension of 0.1 mL (approximately 5 × 10⁶ cells) was injected subcutaneously into the right shoulder blade of mice.²⁴ After achieving 100 mm³ of tumor volume, mice (*n* = 8) were treated with vehicle and D-limonene (0.5 mg/kg, 1.0 mg/kg and 1.5 mg/kg) orally for 14 days and tumor growth was observed at the site of injection. Tumor volume was measured externally by digital caliper using following formula:²⁵

$$\text{Volume (mm}^3\text{)} = \frac{A \times B^2}{2}$$

where A was the largest and B the smallest diameter (mm).

At the end of the study, the tumors were excised and histopathological analysis of excised tumors was performed.

3.8. Histopathological analysis

All mice were sacrificed by cervical dislocation. The tumors were excised and maintained in 10% neutral buffered formalin and stained with hematoxylin and eosin. The slides were observed at 100× and 400× magnification and photodocumented by optical microscopy (IX 51; Olympus, Tokyo, Japan) equipped with a digital camera (TL4) for confirmation of presence of tumor cells.

3.9. Statistical analysis

All data were represented as mean + SD. Statistical analysis was performed by one-way ANOVA followed by Bonferroni comparison using Graphpad Prism software (v. 5.0, Graph Pad software Inc, USA). *P* value of 0.05 and 0.001 or less was considered to be statistically significant.

4. RESULTS

4.1. Effect of D-limonene on viability of K562 cells

MTT assay of D-limonene at concentration of 1 mM, 2 mM, 4 mM and 8 mM shows significant reduction percentage of cell viability of K562 cells as compared to untreated cells at 24 h and 48 h of treatment. However, no significant difference was observed in reduction of percentage of cell vi-

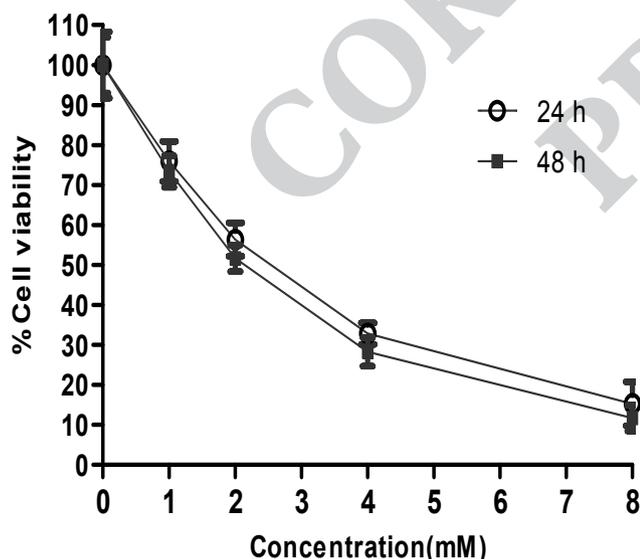


Figure 1. K562 cells were exposed to varying concentrations of D-limonene for 24 h and 48 h. The percentage of cell viability was measured by MTT assay. Cell viability was calculated as a percentage of untreated cells (100%). Data shown are the means ± SD from 3 separate experiments in quadruplicate.

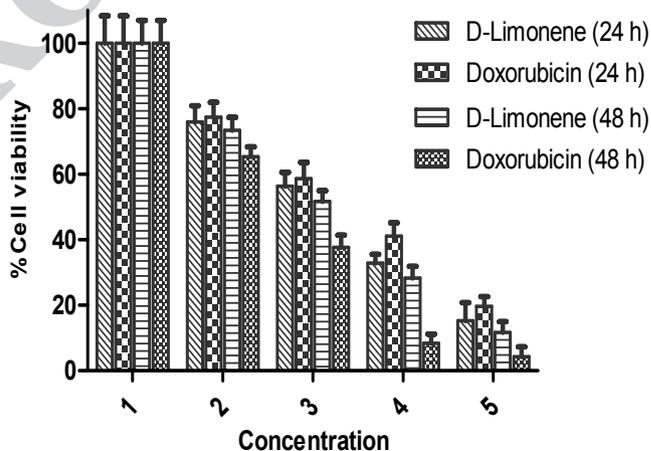


Figure 2. K562 cells shows reduction in percentage of cell viability after treatment with D-limonene and doxorubicin for 24 h and 48 h. D-limonene was used at concentration of 1 mM, 2 mM, 4 mM and 8 mM and doxorubicin was used at concentration of 0.4 μM, 0.8 μM, 1.6 μM and 3.2 μM. The percentage of cell viability was measured by MTT assay. Data shown are the means ± SD from 3 separate experiments in quadruplicate.

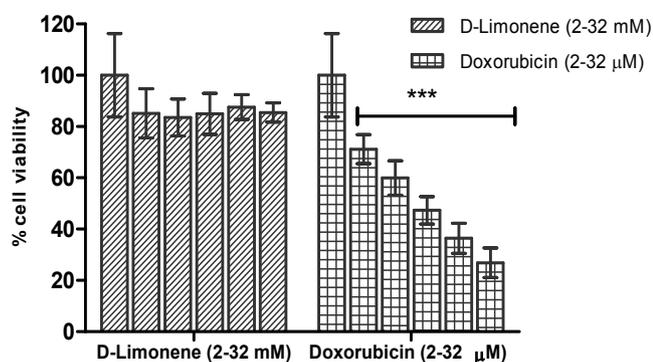


Figure 3. MTT assay of primary hepatocytes from mouse shows reduction in percentage of cell viability after treatment with doxorubicin at concentration of 0 mM, 2 mM, 4 mM, 8 mM, 16 mM and 32 mM. No significant reduction in % of cell viability was observed with D-Limonene at concentration of 2 mM, 4 mM, 8 mM, 16 mM and 32 mM. Data shown are the means \pm SD from 3 separate experiments in quadruplicate.

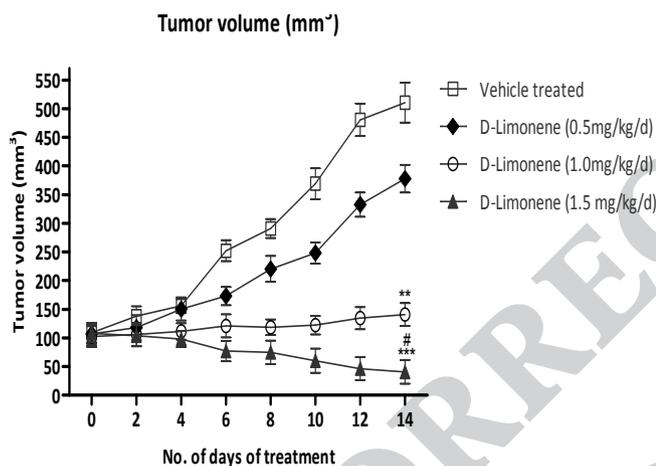


Figure 4. Graph shows the mean tumor volume of K562 xenograft. D-limonene treatment suppresses the tumor growth. K562 xenograft mice were treated with vehicle and with D-limonene at concentration of 0.5 mg/kg a day, 1 mg/kg a day and 1.5 mg/kg a day orally for 14 days. A tumor growth curve was recorded every 2 day. Comments: Mean \pm SD ($n = 8$); * $P < 0.05$ vs. vehicle treated group; # $P < 0.05$ vs. D-limonene (0.5 mg/kg a day) group. No treatment was given to the vehicle treated group.

ability of K562 cells at 24 h and 48 h of treatment. The IC₅₀ value of D-limonene was 3.6 mM and 3.29 mM for 24 h and 48 h of treatment respectively (Figure 1). The reduction in percentage of cell viability of doxorubicin at concentrations of 0.4 μ M, 0.8 μ M, 1.6 μ M and 3.2 μ M was comparable with D-limonene at 24 h and 48 h of treatment (Figure 2). The IC₅₀ value of doxorubicin was 1.6 μ M and 0.95 μ M at 24 h and 48 h, respectively.

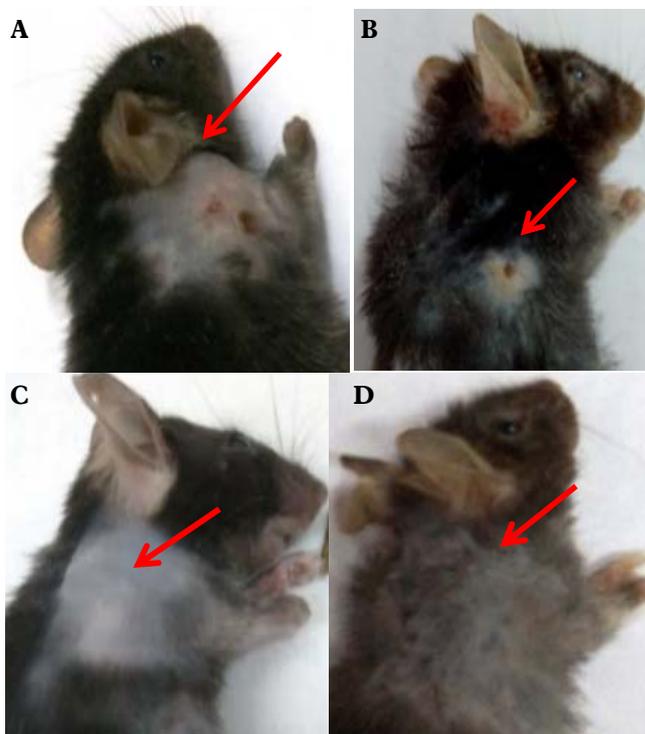


Figure 5. Immunocompromised C57BL/6 mice having K562 tumor xenograft as shown by arrow. Mice were treated with different concentrations of D-limonene, shows significant dose dependent regression of tumor. (A) Vehicle treated, (B) D-limonene 0.5 g/kg a day, (C) 1.0 g/kg a day and (D) 1.5 g/kg a day orally for 14 days. No treatment was given to the vehicle treated group.

4.2. Effect of D-limonene on cell viability of primary mouse hepatocytes

Treatment with D-limonene at concentration of 2 mM, 4 mM, 8 mM, 16 mM and 32 mM for 48 h did not show any reduction in percentage of cell viability of normal mouse primary hepatocytes compared to untreated cells by MTT assay. However, doxorubicin at concentration of 2 μ M, 4 μ M, 8 μ M, 16 μ M and 32 μ M for 48 h shows significant reduction in percentage of cell viability (IC₅₀ = 14.13 μ M) of mouse primary hepatocytes as compared to untreated cells (Figure 3).

4.3. Tumor xenograft model shows regression of the malignant tumor

Immunocompromised K562 cell injected C57BL/6 mice showed palpable tumor on 3rd day of tumor cell implantation. Increase in mean tumor volume was seen with the vehicle treated group every day. D-limonene treatment at daily dose of 0.5 mg/kg, 1.0 mg/kg and 1.5 mg/kg for 14 days shows dose dependent reduction in mean tumor volume as compared to vehicle treated group (Figure 4).

However, significant reduction in tumor volume was observed only at dose of 1.0 mg/kg a day and 1.5 mg/kg a day with D-limonene treatment as compared to vehicle treated group. The reduction in tumor size was observed in immu-

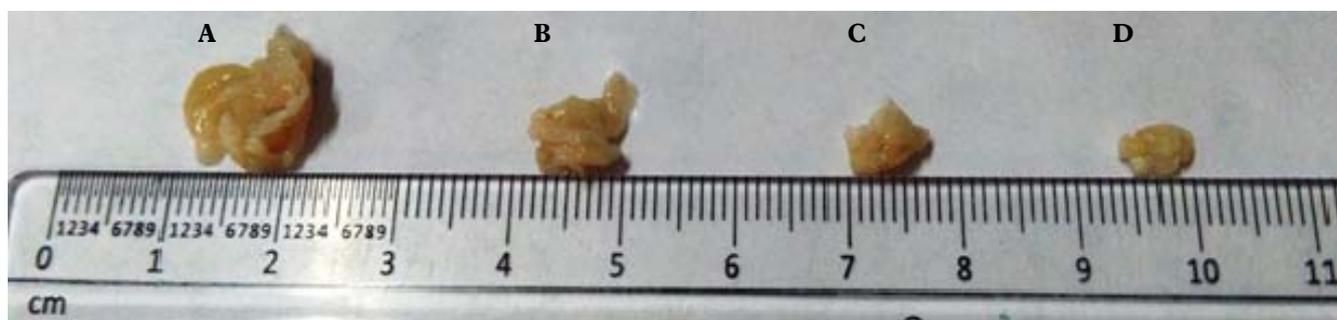


Figure 6. Tumors were excised at the end of 14 days from K562 tumor implanted xenograft C57BL/6 mice. (A) Vehicle treated, (B) D-limonene 0.5 g/kg a day, (C) 1.0 g/kg a day and (D) 1.5 g/kg a day orally for 14 days. D-limonene treated group at concentration 1.5 g/kg a day, shows more reduction in the tumor size as compared to 0.5 g/kg a day and 1.0 g/kg a day. No treatment is given to vehicle treated group.

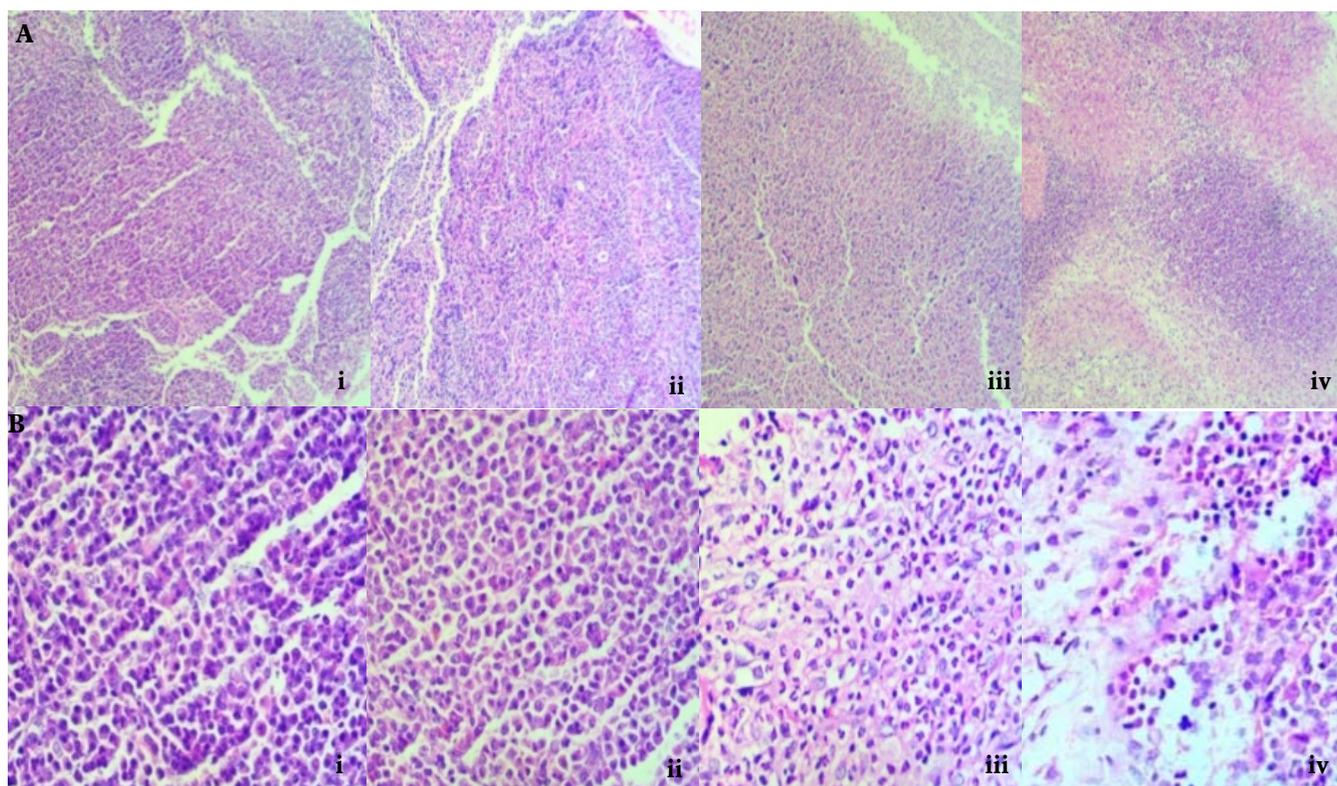


Figure 7. Light microscopy observation of HE-stained section of K562 xenograft tumor at (A) 100 \times and (B) 400 \times magnification. (i) Vehicle treated, (ii) D-Limonene 0.5 g/kg a day, (iii) 1.0 g/kg a day and (iv) 1.5 g/kg a day orally for 14 days. Cells with large and irregular nuclei and scant cytoplasm are characteristic of malignant cells.

nocompromised C57BL/6 mice with D-limonene in dose dependent manner at the end of 14 days treatment (Figure 5). The weight of excised tumors was significantly reduced in the D-limonene treatment groups, compared to vehicle-treated group in dose dependent manner (Figure 6).

4.4. Histopathological analysis

Histopathological data shows the presence of malignant tumor when stained with standard hematoxylin and eosin. The stained section of K562 xenograft shows a characteristic of malignant cells, large and irregular nuclei and scant cytoplasm at magnification of 100 \times and 400 \times (Figure 7). It also shows invasion of malignant cells in adjacent stromal tissue.

5. DISCUSSION

Terpenes have been reported for its multiple biological effects, including antiviral, antibacterial, anti-inflammatory, antioxidant and anti cancer activity. D-limonene, a monocyclic monoterpene obtained from citrus fruits, has shown in vitro cytotoxic effect on various cancer cell lines along with K562 cells.^{14,15} It has also inhibited DMBA-NMU induced mammary carcinoma in rats.^{26,27}

Phase I clinical trials in patients with advanced cancer, D-limonene has shown well tolerated and proven clinical activity.²⁸ D-limonene in combination with docetaxel has improved the sensitivity of hormone refractory prostate

cancer cells, without producing toxicity to normal prostate epithelial cells.^{29,30} The combined beneficial effect could be through the modulation of proteins involved in mitochondrial pathway of apoptosis.

In our study, the cell viability assay of D-limonene has shown significant reduction in the percentage of cell viability in concentration dependent manner which suggests its cytotoxic action on K562 cell line. Anthracycline antibiotic, doxorubicin is one of the most common and widely used anticancer agents and is still a milestone in the therapy of many carcinomas. Similarly with available anticancer treatment, doxorubicin therapy is mostly accompanied by severe side effects based on its systemic toxicity, especially cardiotoxicity and hepatotoxicity.^{31–33} In our study, we have evaluated the effect of D-limonene on isolated normal primary hepatocytes from mice by using doxorubicin as a standard. Doxorubicin showed significant reduction in percentage of cell viability of primary hepatocytes in a concentration dependent manner. Treatment with D-limonene did not show significant reduction in percentage viability of primary hepatocytes. This may be attributed to its antioxidant activity.³⁴

Previous studies suggest, D-limonene shows regression of tumor in in-vivo animal models.^{19,20} Immunocompromised K562 tumor xenograft model was used to evaluate the in vivo efficacy of D-limonene at different doses. Immunosuppression was achieved with combination treatment of cyclosporine, ketoconazole and cyclophosphamide in C57BL/6 mice with 100% take rate.²⁴ Significant increase in tumor volume was observed after 2–3 days of K562 cell implantation. D-limonene treatment showed significant reduction in tumor volume in K562 tumor xenograft mice as compared with vehicle treated group in dose dependent manner. The significant reduction in tumor weight of excised tumors was also observed with D-limonene treatment.

The histological studies of tumor tissue confirmed the presence of malignant tumor cells. It also showed invasion of tumor cells into adjacent stromal tissue. However, the density of the malignant cells was reduced in the dose dependent manner with D-limonene treatment.

6. CONCLUSIONS

In conclusion, the results from the present in vitro study suggests that D-limonene do not show toxicity on normal primary hepatocytes isolated from mouse as compared to doxorubicin. The in vivo studies on K562 tumor implanted C57BL/6 mice, D-limonene shows regression of tumor growth in dose dependent manner. Further studies need to elucidate the mechanism of D-limonene in CML before its clinical application.

Conflict of interest

None.

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