



## SHORT COMMUNICATION

# Anti-leukemic and anti-angiogenic effects of D-Limonene on K562-implanted C57BL/6 mice and the chick chorioallantoic membrane model

Bhavini B. Shah<sup>1</sup>  | Ruma Baksi<sup>2</sup> | Kiranj K. Chaudagar<sup>3</sup> | Manish Nivsarkar<sup>2</sup> | Anita A. Mehta<sup>1</sup>

<sup>1</sup>Department of Pharmacology, L. M. College of Pharmacy, Ahmedabad, Gujarat, India

<sup>2</sup>Department of Pharmacology and Toxicology, B. V. Patel Pharmaceutical Education and Research Development Centre, Ahmedabad, Gujarat, India

<sup>3</sup>Department of Surgery, University of Chicago, Chicago, Illinois

**Correspondence**

Anita Mehta, Department of Pharmacology, L. M. College of Pharmacy, Navrangpura, Ahmedabad, Gujarat, India.  
Email: dranitalmcp@gmail.com

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**Abstract**

**Background:** D-Limonene, a monoterpene from citrus fruit has been found to have chemopreventive and chemotherapeutic activities in various types of cancers. In this study, we evaluated the in vivo effect of D-Limonene on a K562-induced model of chronic myeloid leukemia (CML) in C57BL/6 mice.

**Method:** The tail vein injection model of K562 cells in immunocompromised C57BL/6 mice was developed and evaluated for characteristics of the disease. The mice were treated with D-Limonene and evaluated for haematological parameters. We also evaluated the effect of D-Limonene on angiogenesis using the chick chorioallantoic membrane (CAM) assay.

**Results:** In a complete blood count, a significant dose-dependent reduction in white blood cell, neutrophil and lymphocyte counts, but an elevation in red blood cell count and haemoglobin content was observed with D-Limonene treatment compared to the disease control or untreated group. In the CAM assay, D-Limonene produced a significant dose-dependent reduction in number of blood vessels in treatment groups compared to the vehicle-treated group.

**Conclusion:** These studies suggest promising anti-leukemic and anti-angiogenic effects of D-Limonene in the treatment of CML.

**KEYWORDS**

angiogenesis, C57BL/6 mice, chick chorioallantoic membrane, chronic myeloid leukemia, D-Limonene

## 1 | INTRODUCTION

Chronic myeloid leukemia (CML) is a myeloproliferative disorder characterized by the presence of the Philadelphia chromosome. Reciprocal translocation t(9;22)(q34;q11) generates the Bcr-Abl chimeric gene which leads to constitutive activation of the tyrosine kinase domain.<sup>1</sup> First line treatment with tyrosine kinase inhibitors

(TKIs) has an excellent response in CML patients, with improved life expectancy. However, resistance to a currently used TKI, imatinib, has developed in the chronic (35%), accelerated (45%) and blastic (90%) phases of the disease. This is associated with a poor prognosis in CML patients, and has increased the need for new drug for the treatment of the disease. Natural products are now rapidly emerging

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and appear promising for the development of new anti-cancer drugs.<sup>2,3</sup>

D-Limonene, a monocyclic monoterpene present in citrus fruits, has been found to have chemopreventive and chemotherapeutic activity against various types of cancers.<sup>4-9</sup> Oral administration of D-Limonene inhibited the growth of rodent pancreatic, mammary, and gastric carcinogenesis and exhibited anticancer activity.<sup>7,8,10-13</sup> In our previous studies, we reported that D-Limonene inhibited the growth of K562 cells *in vitro* without producing any toxicity in primary hepatocytes isolated from the mouse.<sup>14</sup> In this study, we evaluated the *in vivo* effect of D-Limonene in immunocompromised C57BL/6 mice.

The chick embryo model offers a number of unique advantages for studying the complex, multistep process of tumor cell metastasis. It serves as a naturally immunodeficient host capable of sustaining grafted tissues and cells, without restrictions, because of its underdeveloped lymphoid system.<sup>15,16</sup> The chorioallantoic membrane (CAM) provides a uniquely supportive environment for primary tumor formation, and is a source of angiogenesis, the formation of new blood vessels from an existing vasculature.<sup>17</sup> Angiogenesis has a crucial role in growth, metastasis and dissemination of tumors.<sup>18</sup> The imbalance between pro- and antiangiogenic factors causes pathogenesis and progression of hematologic neoplasias.<sup>19</sup> Increases in a number of angiogenic factors can significantly increase the number of vessels in the bone marrow of patients with CML.<sup>20,21</sup> The present study aimed to evaluate the effect of D-Limonene on angiogenesis using an *in vivo* chick CAM assay.

## 2 | MATERIALS AND METHODS

### 2.1 | Materials

D-Limonene (MP Biomedicals, Solon, OH, USA); RPMI-1640 and fetal bovine serum (FBS) (Himedia, Mumbai, India); antibiotic-antimycotic solution (Gibco, Grand Island, NY, USA); cyclosporine (Sandimmune, Novartis, Basel, Switzerland); ketoconazole (Nizral, Johnson & Johnson, New Brunswick, NJ, USA); cyclophosphamide (Endoxan, Baxter, Halle, Germany); amproxin (Unichem Laboratories Ltd, Mumbai, India); sterilized gelatin sponge (Alinda Healthcare Pvt Ltd, Ahmedabad, India).

### 2.2 | Cell and culture conditions

The human CML cell line (K562) was obtained from NCCS (National Centre for Cell Science, Pune, India).<sup>22,23</sup> Cells were cultured in RPMI-1640 containing 1% antibiotic-antimycotic solution supplemented with 10% FBS and maintained in an incubator (Forma stericycle CO<sub>2</sub> incubator, Thermo Scientific, Waltham, MA, USA) at 37°C with a 5% CO<sub>2</sub> atmosphere.

### 2.3 | Experimental animals and housing conditions

Healthy C57BL/6 (Mahaveera Enterprises, Hyderabad, India) male mice weighing 25-33 g were housed under standard laboratory

conditions (12:12 h light:dark cycle, relative humidity 60 ± 5%, temperature 25 ± 2°C) in individually ventilated cages. The mice were fed with autoclaved balanced rodent food pellets and Ampoxin (0.1 µg/mL in drinking water) was provided *ad libitum*. All the experiments were carried out in strict accordance with guidelines set by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India. The experimental procedures were reviewed and approved by the institutional animal ethics committee (PERD/IAEC/2017/002) prior to initiation of the experiments.

### 2.4 | *In vivo* establishment of K562 cells in C57BL/6 mice and treatment with D-Limonene

Immunosuppression of C57BL/6 mice was achieved with a combination of ketoconazole (10 mg/kg, orally), cyclosporine (30 mg/kg, intraperitoneally) and cyclophosphamide (60 mg/kg, subcutaneously).<sup>24</sup> K562 cells (approx. 5 × 10<sup>7</sup>) were introduced by tail vein injection. Blood was collected weekly from the retro-orbital plexus to confirm the development of the disease. The blood was analyzed for the presence of circulating blast cells by Giemsa staining of a peripheral blood smear and a complete blood count.<sup>25</sup> Following an increase in the number of blast cells in the peripheral blood smear, treatment with D-Limonene (0.5, 1.0 and 1.5 mg/kg, n = 10) orally for 17 days was initiated in all groups except the disease control or untreated group. Body weight and hematological parameters (total white blood cells count [WBC], neutrophil count [NEU], lymphocytes count [LYM], red blood cells count [RBC], hemoglobin content [Hb] and platelet count) were analyzed.

### 2.5 | Chick CAM assay

Fertilized chick eggs were procured from Jesal Agro Ltd, Gandhinagar, Gujarat, India at the time of experiments. Chick eggs were incubated at 37°C and 60% humidity in an egg incubator (A. P. Poultry Equipments, Hyderabad, Telangana, India) in sterile conditions. On the third day of incubation, eggs were sterilized with isopropyl alcohol and an air sac was made by gently aspirating 2 ml albumin, which is enough to bring the membrane down.<sup>15</sup> A small window of 2 × 2 cm was made in the shell of the egg and the viability of the chick embryos was assessed by monitoring rhythmic contraction of the heart. The window was sealed with transparent adhesive tape and the eggs were incubated in the incubator. On the eighth day, the transparent tape was removed and 1 mm<sup>3</sup> gelatin sponges presoaked with D-Limonene at concentrations of 1, 5 and 10 µg per implant (1 implant per egg, n = 12 for each treatment) or with vehicle were implanted on the CAM of the eggs. The eggs were resealed and returned to the incubator. On the 12th day of incubation, the eggs were observed under the microscope (Olympus) at 10× magnification for counting the number of blood vessels surrounding the gelatin sponge implants.<sup>26</sup>

## 2.6 | Statistical analysis

Statistical analysis was performed by using GraphPad Prism software (version 5.03, Graph Pad Software Inc., La Jolla, CA, USA). Data are presented as means  $\pm$  SEM. Significant differences were determined by one-way ANOVA followed by Bonferroni multiple comparisons.  $P < 0.05$  was considered to be statistically significant.

## 3 | RESULTS AND DISCUSSION

Chronic myeloid leukemia is a condition in which an acquired mutation of genes controls the proliferation and development of cells present in the blood and bone marrow.<sup>27</sup> The Philadelphia chromosome resulting from this mutation has the ability to cause proliferation of myeloblasts.<sup>28</sup> Imatinib and other TKIs are losing their effectiveness in the treatment of CML due to developed resistance. Adjuvant therapy or a combination of drugs may be a better alternative in the treatment of disease.<sup>1,29</sup>

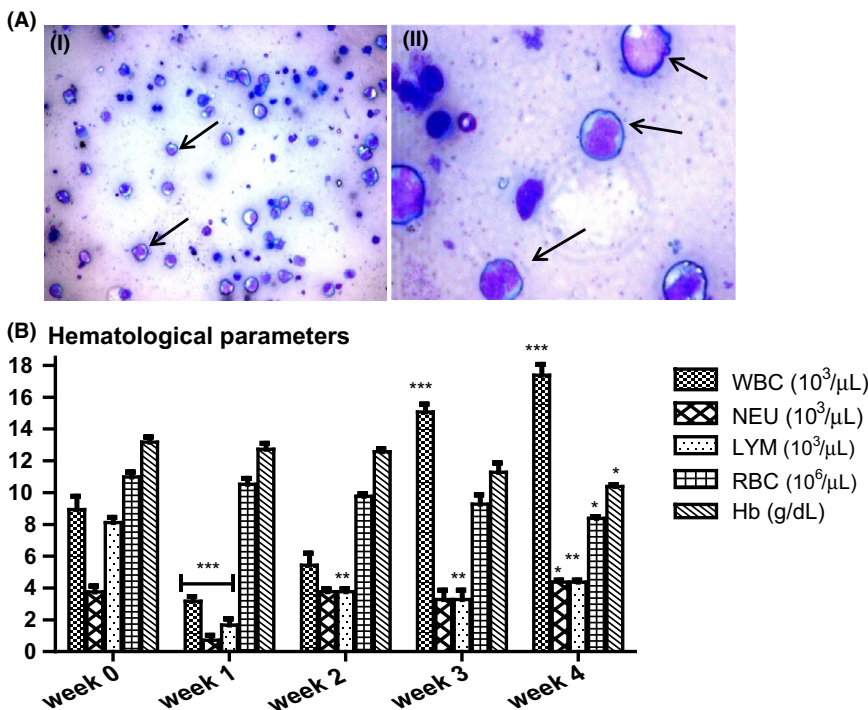
### 3.1 | Determination of model establishment and growth of K562 cells in C57BL/6 mice

We selected the tail vein model among the various available models for CML. Immunosuppression was achieved with ketoconazole, cyclosporine and cyclophosphamide.<sup>24,25</sup> After 1 week of immunosuppression, a significant reduction in WBC, NEU, and LYM counts was noted in a complete peripheral blood count, compared to 0 weeks; however, no significant change was observed in RBC count and Hb content, which confirmed the successful achievement of immunosuppression.

The C57BL/6 mice were injected with K562 cells via tail vein injection followed by immunosuppression and the peripheral blood was examined weekly for circulating blasts using Giemsa staining. The blast cells were differentiated from normal blood cells under the microscope by the larger diameter of the cells ( $\approx 28 \mu\text{m}$ ), the presence of prominent nuclei, reduced cytoplasm and a higher nuclear/cytoplasmic ratio, which confirms the establishment of model.<sup>29</sup> By the fourth week after the transplantation of K562 cells in C57BL/6 mice, Giemsa differential staining of peripheral blood smears showed large numbers of blast cells in the disease control or K562 treated group compared with untreated group.<sup>29</sup> As the disease progressed, changes in haematological parameters were noted. By the fourth week after K562 cell injection, significant increases in WBC, NEU, and LYM counts were observed, compared to 0 weeks, whereas significant reductions in RBC count and Hb content were seen, confirming the establishment of the model (Figure 1). No significant changes in platelet count and other parameters were noted.

### 3.2 | Effect of D-Limonene on the growth of K562 cells in C57BL/6 mice

In CML, the disease progression leads to an increase in the number of granulocytes, which is the reason for the increased numbers of total WBCs, NEUs, and LYMs. However, the significant reduction in RBC count and Hb content is because of a reduced ability to generate new RBCs from bone marrow.<sup>28</sup> D-Limonene treatment at doses of 0.5, 1.0 and 1.5 mg/kg for 17 days in immunocompromised K562 cell-injected C57BL/6 mice produced significant dose-dependent changes in haematological parameters compared to disease control or untreated (DC) group. The treatment also produced a significant



**FIGURE 1** Determination of model establishment and growth of K562 cells in C57BL/6 mice. A, Differential staining of peripheral blood of C57BL/6 mice with Giemsa stain showing the presence of circulating K562 blast cells at 10 $\times$  (i) and 40 $\times$  (ii) magnification, which confirms the establishment of the CML model. B, Haematological parameters show significant reductions in WBC, NEU, and LYM counts 1 wk after tail vein injection of K562 cells compared to week 0. No significant changes were observed in RBC count and Hb content. Four weeks after tail vein injection, significant increases in WBC, NEU, and LYM counts and reductions in RBC count and Hb content were noted compared to week 0. Data shown are the means  $\pm$  SEM ( $n = 10$ ), \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$  vs week 0

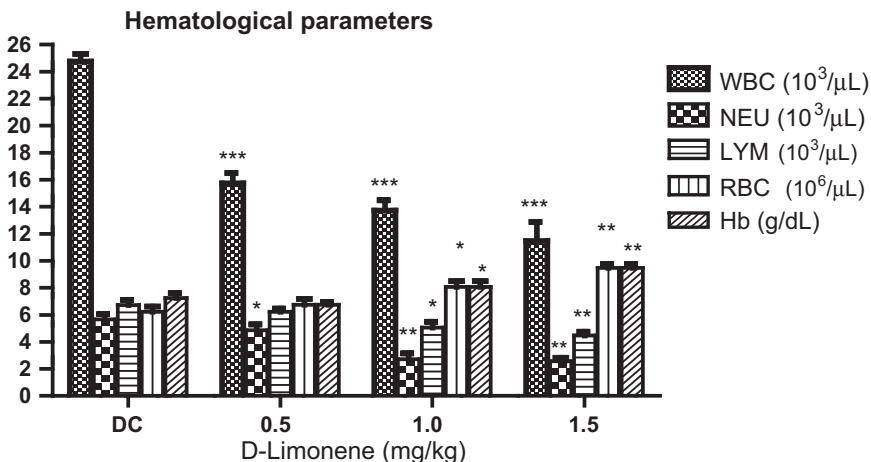
reduction in WBC and NEU counts compared to the DC group. The change in LYM count was not significant with 0.5 mg/kg D-Limonene, but at doses of 1.0 and 1.5 mg/kg a significant dose-dependent reduction was observed compared to the DC group. Changes in RBC count and Hb content were non-significant with D-Limonene treatment (Figure 2). D-Limonene at a dose of 1.5 mg/kg produced the greatest effect on disease progression by reducing WBC, NEU, and LYM counts and increasing RBC count and Hb content

compared to other treatment group, which suggests it has potential for use in the treatment for CML.

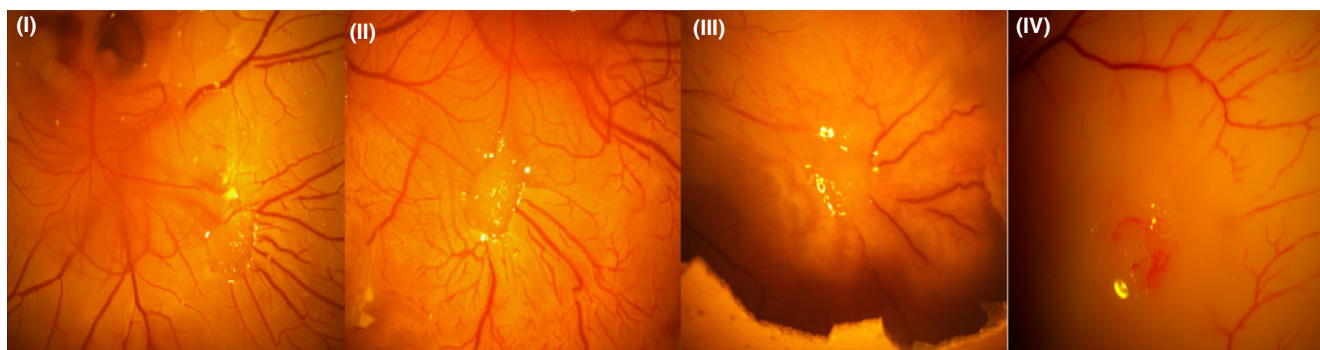
### 3.3 | Effect of D-Limonene on angiogenesis using the CAM assay

Angiogenesis has been linked with the growth, dissemination, and metastasis of various tumors. Angiogenesis plays an important role

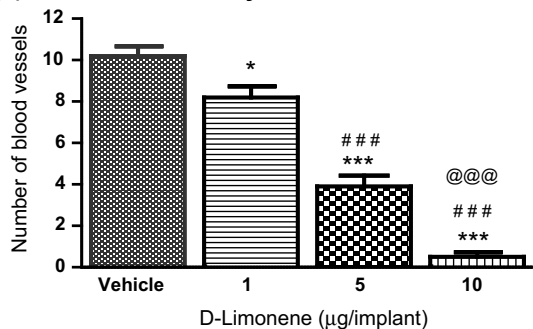
**FIGURE 2** D-Limonene treatment in immunocompromised K562-implanted C57BL/6 mice produced significant changes in haematological parameters. D-Limonene at a dose of 0.5 mg/kg significantly decreased the WBC and NEU counts, whereas RBC count and Hb content were non-significantly increased. At doses of 1.0 and 1.5 mg/kg D-Limonene significantly reduced WBC, NEU, and LYM counts and increased RBC count and Hb content. Data shown are the means  $\pm$  SEM ( $n = 10$ ), \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$  vs DC



(A)



**(B) CAM assay of D-Limonene**



**FIGURE 3** Effect of D-Limonene on angiogenesis using the CAM assay. A, Images show the reduction in number of blood vessels formed on the CAM membrane at 10 $\times$  magnification. The gelatin sponges were soaked with vehicle (i), and D-Limonene at 1  $\mu\text{g}$  per implant (ii), 5  $\mu\text{g}$  per implant (iii) and 10  $\mu\text{g}$  per implant (iv). B, D-Limonene treatment groups at concentrations of 1, 5, and 10  $\mu\text{g}$  per implant induced significant concentration-dependent reductions in the number of blood vessels compared to the vehicle-treated group. The highest reduction was observed with highest concentration of 10  $\mu\text{g}$  per implant. Values are expressed as means  $\pm$  SEM ( $n = 10$ ). \* $P < 0.05$ , \*\*\* $P < 0.001$  vs vehicle-treated implant, ### $P < 0.001$  vs 1  $\mu\text{g}$  per implant, @@@ $P < 0.001$  vs 5  $\mu\text{g}$  per implant

in the progression of CML by affecting the vascularity of bone marrow or leukemic cells. CAM assays have been widely used to study the effect of various compounds on angiogenesis because of their simplicity, cost-effectiveness and reproducibility.<sup>19</sup> We evaluated the effect of D-Limonene on angiogenesis using the CAM assay.

On the fourth day after implantation of gelatin sponges soaked with D-Limonene at concentrations of 1, 5 and 10 µg per implant, a significant dose-dependent reduction in the number of blood vessels on CAM was seen compared to CAM implanted with vehicle. The reduction in the number of blood vessels on CAM was highest when D-Limonene was applied at 10 µg per implant (Figure 3).

Additional studies will be needed to confirm the effect of D-Limonene on the expression of various angiogenic and anti-angiogenic factors.

## 4 | CONCLUSION

In conclusion, we have confirmed that D-Limonene produces a significant dose-dependent reduction in the progression of CML by reducing the growth of K562 blasts in K562 cells implanted into C57BL/6 mice. In CAM assays, D-Limonene also inhibited angiogenesis in a concentration-dependent manner, which suggests its importance as a promising anticancer therapy for CML.

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## CONFLICT OF INTEREST

None.

## AUTHOR CONTRIBUTIONS

Anita A. Mehta, Manish Nivsarkar, Kiranj Chaudagar, and Bhavini Shah conceived and designed the study. Bhavini Shah and Ruma Bakshi carried out the experimental work and data analysis. All authors contributed to revising the manuscript. All authors gave final approval for publication.

## ORCID

Bhavini B. Shah  <http://orcid.org/0000-0003-3005-8273>

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