

# Anti-lymphoma Activity of *Averrhoa bilimbi* Fruit Extract in Swiss Albino Mice

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## Abstract

**Aim:** To evaluate the effect of *Averrhoa bilimbi* fruit extract against Dalton's ascitic lymphoma (DAL) induced in Swiss albino mice. **Materials and Methods:** DAL cell line was injected via intraperitoneal to Swiss albino mice and was treated with *A. bilimbi* fruit extract for 15 days. Then, the animals were sacrificed on 15<sup>th</sup> day to test hematological parameters, body weight, and tumor volume. The *in vitro* growth inhibition effect of *A. bilimbi* fruit extract on DAL cell line was also studied using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. **Results and Discussion:** The *A. bilimbi* fruit extract showed promising anti-lymphoma activity in Swiss albino mice. Treatment of *A. bilimbi* normalized the hematological parameters significantly (\*\* $P < 0.01$ ) by decreasing the high level of white blood cell and by increasing the levels of red blood cells and hemoglobin count when compared with DAL control mice. The treated mice group with *A. bilimbi* fruit extract resulted in significant ( $P < 0.01$ ) decrease in body weight when compared with the control. The MTT assay also has shown a significant growth inhibition percent (97.96%) for the fruit extract treatment. **Conclusion:** The current study revealed the anti-lymphoma activity of the *A. bilimbi* fruit extract against DAL cell line induced Swiss albino mice.

**Key words:** *Averrhoa bilimbi*, Dalton's ascitic lymphoma cell line, mice weight, tumor volume, hematology, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay

## INTRODUCTION

India is a country with 15 agro climatic zones, in which more than 7000 plants are having medicinal usage in common folk and documented systems of medicine. Plants are used in Ayurveda, Siddha, Unani, and Homoeopathy to treat many diseases. It was reported very earlier that 80% of world inhabitant problems are treated by medicinal herbal drugs.<sup>[1]</sup> *Averrhoa bilimbi* belongs to Oxalidaceae family and traditionally its fruits are used in pickle preparations.<sup>[2]</sup> The leaves, flowers, and fruits are used to treat a cough, pimples, hypertension, diabetes, fever, swellings, inflammation, and to stop rectal bleeding.<sup>[3]</sup> *A. bilimbi* is having minerals such as phosphorous, nitrogen, potassium, and iron.<sup>[4]</sup> It is found throughout Malaysia, Indonesia, Myanmar, Bangladesh, Srilanka, and common in Southeast Asian countries. It has antimicrobial activity<sup>[5]</sup> and antidiabetic property.<sup>[6]</sup> This plant is used in traditional medicine as a cure for a cough, cold, itches, boils syphilis, and hypertension.<sup>[7]</sup> In the Philippines, the leaf is being applied as a paste on itches, swelling, rheumatism, mumps or skin eruptions and used for bites against

poisonous infection.<sup>[8]</sup> Another study has reported that the *A. bilimbi* fruit extract showed cytotoxicity activity in brine shrimp lethality bioassay. They found that the methanolic fruit extract has more cytotoxicity than leaf, and the  $LC_{50}$  of the leaf was found to be 92.51 and for the fruit extract was 12.96.<sup>[6]</sup> In cancer study, the tumor mass increased rapidly and spread throughout the body and eventually caused the death of the organism.<sup>[9]</sup> Dalton's ascitic lymphoma (DAL) is cancer causing cells, which increase the tumor cells in the peritoneal cavity and the body of the organism increases to maximum (tumor increase) and can cause death. Up to now, no *in vivo* antitumor studies of *A. bilimbi* fruit extract against DAL-induced ascitic tumor have not reported. Based on the previous phytochemical study, antimicrobial activity and cytotoxicity activity of fruit extract and ethno traditional use the current investigation of *A. bilimbi* fruit extract against DAL-induced ascetic tumor in Swiss albino mice is designed.

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## MATERIALS AND METHODS

### Collection of plant material

The fresh *A. bilimbi* fruits were collected from Palakkad district Kerala region India. The plant was authenticated by the Botanical Survey of India, Coimbatore, Tamil Nadu, and the authentication number is (BSI/SRC/5/23/2016/TECH). The collected fruits of plant sample were washed thoroughly with running tap water and completely shade dried under room temperature.

### Preparation of extract

The shade dried fruits of the plant were subjected for mechanical size reduction. The powdered material was extracted with methanol by using Soxhlets apparatus, and it was concentrated using vacuum rotary evaporator. The obtained extract was preserved in the freezer for further use.

### Animals

Swiss albino male mice with  $25 \pm 2$  g were obtained from Sri Venkateshwara Enterprises, Bangalore, Karnataka, India. Mice were placed in polyacrylic cages (six mice per cage) and housed under standard laboratory conditions with  $25^\circ\text{C} \pm 2^\circ\text{C}$ . Light and dark cycle was maintained as 12:12 h. Animals are maintained with free access of standard dry pellet diet from Sri Venkateshwara Enterprises, Bangalore, Karnataka, India, and water *ad libitum*. For 15 days, the mice were acclimatized to laboratory conditions. After this, the grouping of animals and commencement of experiment was started. All the animal experiments were carried out with proper approval from the Institute Animal Ethical Committee (IAEC) of Karunya University. (IAEC/KU/BT/15/08).

### Drugs and chemicals

Gum acacia was purchased from Hi-Media (Mumbai, India); Drabkin's solution from Nice Chemicals Pvt. Ltd. (Cochin, India); and ethylenediaminetetraacetic acid from Merck. Methotrexate was purchased from (Mumbai, India). Standard Drug Methotrexate was obtained from the IPCA laboratories. All other chemicals used were of analytical reagent grade.

### Cell line

The DAL cell line was obtained from the National Cancer Centre (Pune, India). Their concentration of cells was determined using a hemocytometer before transplantation. Animals were inoculated with  $1.5 \times 10^6$  cells/mouse. All the animals' experiments were carried out with proper approval from the IAEC of Karunya University.

### Tumor cells of DAL

The DAL cell lines are sustained in mice *in vivo* by intraperitoneal injection ( $1.5 \times 10^6$  cells/mice). After 10-15 days (during Log phase), the ascitic fluid was drawn out from tumor bearing mouse and was injected via i.p. to all the mice of Groups II-V in a concentration of  $1.5 \times 10^6$  cells/mice.

### DAL-induced ascitic tumor studies

Animals were divided into five groups containing 6 mice in each group. The tumor was induced by injecting DAL cell lines ( $1.5 \times 10^6$  cells/mouse) intraperitoneal to the left side of Swiss albino mice. Group I served as normal and received vehicle phosphate-buffered saline (PBS) alone. Group II served as tumor control. Groups III and IV were treated with *A. bilimbi* fruit extract at doses of 10 mg/kg. bw and 20 mg/kg. bw, respectively.<sup>[10]</sup> Group V was treated with standard drug methotrexate at a dose of 3.5 mg/kg b.wt. All the treatments were given via i.p. injection at 24<sup>th</sup> h after DAL tumor inoculation and continued for 14 consecutive days. On 15<sup>th</sup> day, all mice were weighed and sacrificed for tumor evaluation. Hematological parameters such as red blood cells count (RBC), white blood cells (WBC) count, and hemoglobin (Hb) content were estimated. The antilymphoma was assessed from the body weight of mice and the tumor volume.<sup>[11]</sup>

### *In vivo* anti-lymphoma study

#### Body weight

DAL cell line was induced to II to V group of animals ( $1.5 \times 10^6$  cells/mice) except normal. Weight of the mice was measured in the control group and treated group before the start of the experiment and after 15 days of treatment.

#### Tumor volume and weight

On 15<sup>th</sup> day (completion of experiment), all the animals are sacrificed, and the ascetic fluid was collected from the peritoneal cavity. The ascetic fluid was collected in centrifuge tube and weighed to determine the weight and the volume were also measured.

#### Hematological parameters

On 15<sup>th</sup> day of treatment, animals were kept starvation for 1 day and the animals were sacrificed. The blood was collected through cardiac puncture and used for the estimation of total WBC, RBC count, and Hb content.<sup>[11]</sup>

### 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay for *in vitro* anti-lymphoma study

MTT assay is based on calorimetric assay, in which the potent nature of mitochondria succinate dehydrogenase enzyme

in living cells reduce the yellow soluble substrate MTT to formazan which is an insoluble and colored substance.<sup>[12]</sup> About 0.1 ml ascitic tumor bearing DAL cell lines were collected and diluted with PBS. Cells are seeded at a density of  $1.5 \times 10^6$  cells/well (100  $\mu$ l) in a 96 well plate and incubated in CO<sub>2</sub> incubator at 37°C. After 24 h of incubation, *A. bilimbi* fruit extract was added at different concentrations (50, 100, 150, 200, and 250  $\mu$ g) and incubated for 48 h, after incubation MTT solution (0.5 mg/ml) was added and incubated for 4 h at 37°C. Immediately, the absorbance of the solution was measured spectrophotometrically at 590 nm using ELISA microplate reader. All the resulted mean values are obtained by triplicate values of each concentration. The percentage of viable cells was calculated by the following formula.<sup>[13]</sup> From this, the percent growth inhibition of DAL cell line was calculated.

Percent viability (%) =  $\frac{\text{OD of the control (untreated cells)} - \text{OD of the test}}{\text{OD of the control (untreated cells)}} \times 100$

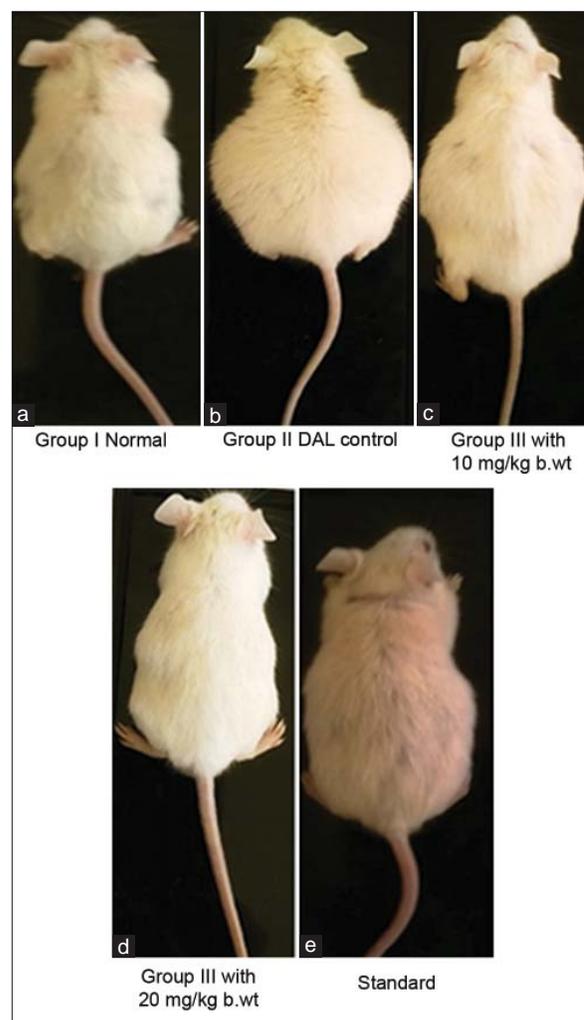
### Statistical analysis

All the experimental values are expressed as mean $\pm$ SD. The statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Dunnett's *t*-test using GraphPad InStat version 3.0, GraphPad Software, San Diego, California, USA. *P* values (i.e., \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001) were considered statistically significant compared to DAL tumor control.

## RESULTS

### Effect of *A. bilimbi* on body weight

Weight of the mice in the control group was increased and in treated groups that are in Group III and Group IV the weight of the animal was decreased. It is indirectly



**Figure 1:** Size of the animal showing difference in their size and the effect of the *Averrhoa bilimbi* fruit extract of Dalton's ascitic lymphoma (DAL)-induced mice (a). Weight of the mice was increased in DAL control mice (b). After treating cancer mice with *A. bilimbi* fruit extract, the weight of the mice was decreased (c and d). Then, standard drug treatment also has shown weight reduction (e)

**Table 1:** Body weight of the mice after and before the treatment of different groups are mentioned

Mice	Before DAL cell line Induced to Swiss albino mice in grams	After DAL cell line induced to Swiss albino mice in grams (On 15 <sup>th</sup> day)
Group I	24.66 $\pm$ 2.08	25.66 $\pm$ 3.51 <sup>a</sup>
Group II	24 $\pm$ 1.09	33.33 $\pm$ 1.36*
Group III	24.83 $\pm$ 0.98	27.5 $\pm$ 1.22 <sup>ab</sup>
Group IV	23.66 $\pm$ 1.21	25.66 $\pm$ 1.21 <sup>ab</sup>
Group V	24.833 $\pm$ 1.16	26.5 $\pm$ 1.04 <sup>ab</sup>

The values represented as mean $\pm$ SD. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001. <sup>a</sup>Group I - Normal versus Group II - DAL control. <sup>b</sup>Treatment Groups III, IV, and V versus Group II DAL control. *A. bilimbi* fruit extract (Groups III and IV) showed the decreased tumor activity in DAL-induced mice when compared with DAL control (Group II). DAL: Dalton's ascitic lymphoma, *A. bilimbi*: *Averrhoa bilimbi*, SD: Standard deviation, Group I is the normal group (untreated group), Group II is a control group (Treated with DAL cell line alone), Group III and Group IV are treated with DAL cell line at 0<sup>th</sup> day and for 14 days treated with the *Averrhoa bilimbi* fruit extract, Group V is treated with DAL cell line at 0<sup>th</sup> day and for 14 days treated with the Standard drug, Group IV showing more significant than the Group V i.e *P* < 0.01 (\*\*). *Averrhoa bilimbi* fruit extract in Group IV showed better results i.e decrease in the body weight.

showing the effect of *A. bilimbi* fruit extract on the cancer cell line. The difference in body weight of the mice before and after the DAL cell line induced was shown in Table 1 and Figure 1.

### Effect of *A. bilimbi* on tumor volume and weight

When ascitic fluid collected on 15 days of treatment from the peritoneal cavity, *A. bilimbi* fruit extract significantly ( $P < 0.01$ ) reduced the tumor volume and weight when compared with that of the control group. The tumor volume and tumor weight were shown in Table 2.

### Hematological parameters

Total WBC count was increased in the control group when compared with the normal, and the WBC count was decreased in Group III and IV. For the standard drug also, the level of WBC count was decreased. RBC count and Hb percentage were decrease in control group and in Group III and IV they decreased, which shows better antitumor activity than the standard drug. RBC count, WBC count, and Hb percent were conducted in triplicates, and mean values are tabulated in Table 3.

### MTT assay

For increased concentration 50, 100, 150, 200, and 250  $\mu\text{g}$  of *A. bilimbi* fruit extract increased inhibition was observed.

All the resulted OD values are taken in triplicates, and mean values are used in the calculation. The percentage of inhibition and percent of viable cells are tabulated in Table 4 and Figure 2.

## DISCUSSION

The current investigation was carried out to find the anti-lymphoma activity of *A. bilimbi* fruit extract in DAL-induced mice as well as against DAL cell lines *in vitro* MTT assay. *A. bilimbi* fruit has a rich source of vitamins, minerals, and acids, which are beneficial to human being.<sup>[14]</sup> The phytochemicals present in it indicate the presence of high quality and quantity of antioxidant capacity.<sup>[15]</sup> *A. bilimbi* is a rich source of phytochemicals with antibacterial and antifungal activity. The fruit of *A. bilimbi* has more cytotoxic activity than the leaf.<sup>[5]</sup> The  $1.5 \times 10^6$  cells were induced to all group mice except normal group. The body weight of the mice was significantly increased in ( $P < 0.01$ ) control group when compared with the normal, which indicates the multiplication of DAL cell line in the control (DAL) group. The treatment with *A. bilimbi* fruit extract to Group III and Group IV mice resulted in significant ( $P < 0.01$ ) decrease in body weight when compared with control. *In vitro* viability of cells was measured by the MTT assay and was found to be 97.96% of inhibition for the 250  $\mu\text{g}$  of the fruit extract. By this, *A. bilimbi* fruit extract showed the tumor inhibition. DAL will form ascetic fluid (tumor) and spread entire peritoneal cavity, and this will

**Table 2: Effect of *Averrhoa bilimbi* fruit extract on the tumor volume and tumor weight**

Parameters	Group II	Group III	Group IV	Group V
Tumor volume in ml	16.16 $\pm$ 0.84	11.88 $\pm$ 0.63**	6.5 $\pm$ 0.73**	5.42 $\pm$ 0.66**
Tumor weight in g	15.33 $\pm$ 1.15	9.9 $\pm$ 0.80**	6.05 $\pm$ 0.62**	4.22 $\pm$ 0.45**

The values represented as mean $\pm$ SD. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . Treatment Groups III, IV, and V were compared to Group II DAL control for statistical significance. *Averrhoa bilimbi* fruit extract (Groups III and IV) showed the decreased tumor activity in DAL-induced mice when compared with DAL control (Group II). SD: Standard deviation, DAL: Dalton's ascitic lymphoma, Group II is a control group (Treated with DAL cell line alone), Group III and Group IV are treated with DAL cell line at 0<sup>th</sup> day and for 14 days treated with the *Averrhoa bilimbi* fruit extract, Group V is treated with DAL cell line at 0<sup>th</sup> day and for 14 days treated with the Standard drug, When group III, IV and V compared with the group II it showed a significant value i.e  $P < 0.01$  (\*\*). Tumor volume and weight in the treated group is less when compared with the control group.

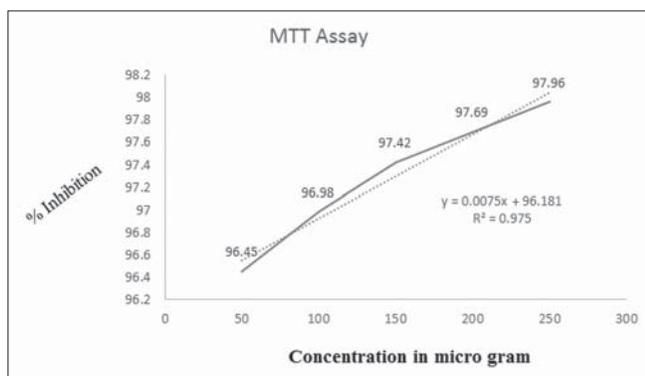
**Table 3: Total WBC count, RBC count, and Hb percentage for the different group of animals**

Hematological parameters	Total WBC count (cells/ml $\times 10^4$ )	RBC count ( $10^9/\mu\text{L}$ )	Hb (%)
Group I	10.52 $\pm$ 1.17	4.83 $\pm$ 0.27	13.08 $\pm$ 0.83
Group II	13.9 $\pm$ 0.88	2.63 $\pm$ 0.30	5.76 $\pm$ 0.47
Group III	11.6 $\pm$ 0.45**	3.87 $\pm$ 0.06**	7.7 $\pm$ 0.39*
Group IV	10.62 $\pm$ 0.48**	3.26 $\pm$ 0.13**	9.3 $\pm$ 0.44**
Group V	11.35 $\pm$ 0.47**	3.68 $\pm$ 0.18**	9.2 $\pm$ 0.45*

All the data were expressed as mean $\pm$ SD. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  extract treated groups compared with the Group II. WBC: White blood cells, RBC: Red blood cells, Hb: Hemoglobin, SD: Standard deviation, Group I is the normal group (untreated group), Group II is a control group (Treated with DAL cell line alone), Group III and Group IV are treated with DAL cell line at 0<sup>th</sup> day and for 14 days treated with the *Averrhoa bilimbi* fruit extract, Group V is treated with DAL cell line at 0<sup>th</sup> day and for 14 days treated with the Standard drug, Due to the treatment of DAL cell line to control group increased the WBC count, decreased the RBC count and decreased the Hb %. By treating the groups III and IV with *Averrhoa bilimbi* fruit extract, it decreased the WBC count and increased the RBC count and Hb %, Group III and IV shown a significant value of  $P < 0.01$  (\*\*). Maintained the optimum values for the WBC count, RBC count and HB %.

**Table 4:** Percentage of inhibition for the *Averrhoa bilimbi* fruit extract

Concentration (µg)	Percentage of inhibition by the <i>Averrhoa bilimbi</i> fruit extract (%)
50	96.45
100	96.98
150	97.42
200	97.69
250	97.96

**Figure 2:** Representing the percentage of Inhibition of *Averrhoa bilimbi* fruit extract for different increased concentration

affect the liver normal function.<sup>[16]</sup> In our study, we observed an increased tumor volume and weight in control which was significantly reduced in the treated group. In the control (DAL) group, increased the level of total WBC count and decreased the level of RBC count were observed. Treatment of *A. bilimbi* decreased the high level of WBC and increased level of RBC count significantly (\*\* $P < 0.01$ ). Hb percentage in control was reduced due to DAL and in treated group the elevated level of Hb percentage was observed.

Plant-based natural products such as phenols, flavonoids, terpenoids, saponins, and steroids have received considerable attention due to their diverse pharmacological properties such as antioxidant and antitumor activity.<sup>[17]</sup> Antioxidants play an important role in inhibiting and scavenging radicals, thus providing protection to humans against infection and degenerative diseases. Majorly these compounds have a chemopreventive role in cancer through their effects on signal transduction in cell proliferation and angiogenesis.<sup>[18]</sup> Ellagic acid in a polyphenol found in fruits such as *Raspberries* and *Strawberries*,<sup>[19]</sup> which act as detoxifying agent by binding to carcinogen and make them inactive<sup>[20]</sup> and ellagic acid prevent the binding of carcinogens to DNA and reduces the cancer risk.<sup>[21]</sup> Apigenin is a flavonoid found in parsley, artichoke, basil, celery and it induces the apoptosis.<sup>[22]</sup> Apigenin inhibits cancer cell signal transduction and induces the apoptosis. It is the best source for treating human anaplastic thyroid carcinoma.<sup>[23]</sup> The epidermal growth factor signal transduction pathway is an essential component of both

cancer cell growth and differentiation. Apigenin interferes with the epidermal growth factor cell stimulation and reduces the risk of colorectal cancer by interfering with the cancer cell growth signaling pathway.<sup>[24]</sup> Preliminary phytochemical analysis revealed the *A. bilimbi* fruit has a rich source of phytochemicals<sup>[25]</sup> such as phenols, flavonoids, alkaloids, and saponins. The fruits have more cytotoxicity levels than leaves.<sup>[5]</sup> The leaves and fruit have antimicrobial, antifungal activity, and antidiabetic activity.<sup>[26]</sup> However, the specific phytochemicals present in this extract need to be analyzed.

Due to the pharmacological activity, current investigation was performed to check the anticancer activity of *A. bilimbi* fruit extract against DAL-induced Swiss albino mice. The fruit extract showed the anticancer activity both in *in vitro* and *in vivo* models.

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