Anti-tumor and Chemoprotective Effects of *B. tomentosa*

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Abstract

Cancer is a leading cause of death worldwide. Due to the toxic side effects of the commonly used chemotherapeutic drug cyclophosphamide (CTX), the use of herbal medicines with fewer side effects but having potential use as inducing anti-cancer outcomes *in situ* has become increasingly popular. The present study sought to investigate the effects of a methanolic extract of *Bauhinia tomentosa* against Dalton’s ascites lymphoma (DAL) induced ascites as well as solid tumors in BALB/c mice. Specifically, *B. tomentosa* extract was administered intraperitoneally (IP) at 10 mg/kg BW body weight starting just after tumor cell implantation and thereafter for 10 consecutive days. In the ascites tumor model hosts, administration of extract resulted in a 52% increase in the life span. In solid tumor models, co-administration of extract and CTX significantly reduced tumor volume (relative to in untreated hosts) by 73% compared to just by 52% when the extract alone was provided. Co-administration of the extract also mitigated CTX-induced toxicity, including decreases in WBC count, and in bone marrow cellularity and α-esterase activity. Extract treatment also attenuated any increases in serum levels of TNFα, iNOS, IL-1β, IL-6, GM-CSF, and VEGF seen in tumor-bearing hosts. This study confirmed that, the potent antitumor activity of *B. tomentosa* extract may be associated with immune modulatory effects by regulating anti-oxidants and cytokine levels.

Keywords: *Bauhinia tomentosa* - anti-tumor activity - cyclophosphamide - Dalton’s ascites lymphoma.

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Introduction

Cancer is one of the leading cause of death among humans and is characterized by uncontrolled growth and spread of abnormal cells. Scientific investigations are making best efforts to combat this disease, but the sure-shot, perfect cure is yet to be brought into world medicine. There are several external factors (tobacco, chemicals and radiation) and internal factors (inherited mutations, hormones etc.) leading in the initiation or promotion of carcinogenesis (Grivennikov et al 2010). There are several therapies available to treat cancer and the major one among them is radiotherapy and chemotherapy. Even though these therapies have shown promising results, some of the major drawbacks of this therapy are toxic side effects and suppression of the immune system. (Diwanay et al., 2004).

Cyclophosphamide (CTX) belongs to a group of alkylating agents used widely to treat several types of cancer and autoimmune diseases (Haque et al., 2003). CTX has been reported to disturb fundamental mechanisms concerned with cell growth differentiation and function (Alenzi et al., 2010), this chemotherapeutic has also been reported to induce several toxic side effects like nausea, fatigue, hair loss, and to cause reduction in host levels of immune cells (Kiuchi et al., 2009). Due to the toxic side effects of CTX, the need for new drugs effective against solid tumors is an important and necessary strategy to improve the arsenal of agents that could be used during chemotherapy. There has been growing interest in alternative therapies and therapeutic use of natural products, especially those derived from plants (Sakthivel and Guruvayoorappan, 2013). Of the latter class, only a small percentage (10%) has been investigated phytochemically and an even smaller percentage properly studied in terms of pharmacological properties (Rates, 2001). Natural products represent over 50% of all drugs in clinical use and about 85% of traditional medicine involves use of plant extracts; many have been reported to possess several pharmacologically important components. *Bauhinia tomentosa* (family: Caesalpiniaceae) is a medicinal plant widely distributed in tropical/subtropical regions. Many Bauhinia species are rich in β-sitosterol, lupeol, ascorbate (vitamin C), campferol, flavonone, quercetin, etc. and have been shown to impart anti-tumor, -ulcer, -bacterial, and -fungal effects (Rajkapoor, 2006). *Bauhinia tomentosa* has been reported to impart anti-oxidant (Mannangatti et al., 2010), -diabetes (Devaki et al., 2011), and -microbial effects (Gopalakrishnan and Vadivel, 2011). Preparations of *B. tomentosa* have been shown to stimulate the immune system and act as potential anti-inflammatory agents (Kannan et al., 2010). Aderogha et al. (2008) reported the ethanolic extract of *B.

Tumor cells

Dalton Ascites Lymphoma (DAL) cells were obtained from the Amala Cancer Research Institute Thirissur, India. The DAL cells were maintained in vivo in normal Balb/c mice by intra-peritoneal (IP) inoculation of 10⁶ cells into a new mouse after every 10 days.

Assessment of effect of B. tomentosa extract on DAL-induced ascites and tumor formation

The mice were randomly allocated into four groups (n=15/group). Mice in Group I were to serve as a normal control and to be treated with saline. Mice in Group II were to serve as DAL only controls. Mice in Group III were to be treated with the DAL and also with the B. tomentosa extract (at 10 mg/kg, BW). Mice in Group IV were to be treated with DAL and also with methotrexate (at 3.5 mg/kg, BW). DAL cells were collected from donor mice (on Day 9 after initial injection), suspended in sterile saline, and their viability and concentration determined via trypan blue exclusion. Except for mice in Group I, all mice were injected IP with 1.5 x 10⁶ DAL cells; this was designated as Day 0. The respective treatments were to continue daily up to 10 days post-injection of the DAL cells. Body weights of the mice were recorded at the beginning of the experiment (Day 0) and every 5 days over the course of the treatment period.

On Days 10 and 15, subsets of mice (n=6) from each group were euthanized by cervical dislocation and ascites fluid was recovered; blood was also collected (by cardiac puncture) to permit estimation of hemoglobin content (Hb) and white blood cell (WBC) levels. Other samples of the blood were processed to yield serum for subsequent analyses of various parameters (see below). The remaining three mice in each group were maintained to monitor lifespan. Mean survival times across the treated groups were compared with the DAL control group using the calculation Mean Survival Time (MST)=(Day of first death + Day of last death)/2. The Percentage Increase in Lifespan (% ILS), a reflection of anti-tumor efficacy of a given treatment, was calculated as 100 x [(MST of treated group) - (MST of DAL control group)]/MST of DAL control group (Huang et al., 1984).

Effect of extract against DAL-induced solid tumor and cyclophosphamide (CTX) toxicity

Another set of mice was randomly allocated into seven groups (n=18/group). Mice in Group I were to serve as a normal control and to be treated with saline. Mice in Group II were to be treated with CTX only (25 mg/kg, BW). Mice in Group III were to be treated only with 25 mg CTX/kg BW and B. tomentosa extract (10 mg/kg, BW). Mice in Group IV were to serve as DAL only controls. Mice in Group V were to be treated with the DAL and then with 25 mg CTX/kg. Mice in Group VI were to be treated with the DAL and then with 25 mg CTX/kg and B. tomentosa extract (10 mg/kg, BW). As before, DAL cells were collected from donor mice, suspended in sterile saline, and their viability/concentration determined via trypan blue exclusion. Except for mice in Groups I-III, all other mice

Materials and Methods

Preparation and administration of plant extract

B. tomentosa leaves were collected from Coimbatore, India. The plant was identified and authenticated at the Botanical Survey of India, Coimbatore (No: BSI/SRC/5/23/2011-12-Tech-756). Air-dried whole plants of B. tomentosa were powdered and extracted (10g) overnight in 100 ml of 70% methanol with stirring. Supernatant was collected after centrifuging at 5,000 rpm for 10 min. Methanol was removed by evaporation and yield of the extract was 12% (w/w). For in vivo studies, a fixed amount of the dried extract was suspended in 1% gum acacia to provide a solution that would yield a dosage of 10 mg/kg, BW, body weight via intraperitoneal injection (100 µl/dose). This dose was selected according to a cytotoxicity study of B. tomentosa extract reported by Deveki et al. (2011).

Chemicals

Cyclophosphamide (CTX; LEDOXAN™) was obtained from Dabur Pharma Ltd. (New Delhi, India). DTNB (5,5’-dithiobis-2-nitrobenzoic acid) was purchased from SISCO Research Labs (Mumbai, India). Alanine aminotransferase (ALT), aspartate aminotransferase (AST), urea, and creatinine analyzing kits were obtained from SPAN Diagnostics Ltd. (Surat, India). All other chemicals used in the study were of analytical reagent grade. γ-glutamyl transpeptidase kit was purchased from MerckTM, Mumbai, India. Highly specific quantitative sandwich ELISA kits specific for evaluating levels of mouse interleukin (IL)-1β, IL-6, IL-2, GM-CSF, VEGF, and interferon (INF)-γ were procured from Koma Biotech™, (Seoul, Korea). Kits for measuring tumor necrosis factor (TNF)-α and iNOS were bought from USCN Life Sciences (Hubei, China). The limit of sensitivity of the IL-1β, IL-6, IL-2, TNFα, iNOS, VEGF, IFNγ, and GM-CSF kits were, respectively, 15, 5, 4, 3, 6.5, 23, 23 and 8 pg/ml.

Animals

Adult male Balb/c mice (4-6-wk-of-age, 23-25g) were purchased from Kerala Veterinary and Animal Sciences University (Manathy, India). The animals were kept under specific pathogen-free conditions in facilities maintained at 24 ±2°C and a 50% relative humidity and with a 12-hr light:dark cycle. All mice were provided ad libitum access to normal mouse chow (Sai Durga Feeds, Bangalore, India.) and filtered water. All the animal experiments were performed after obtaining permission (IAEC/KU/BT/13/08) from the Institutional Animal Ethics Committee, Karunya University.

were injected IP with 1.5 x 10⁶ DAL cells on Day 0. The respective treatments were to continue daily up to 10 days post-injection of the DAL cells.

Body weights of the mice were recorded at the beginning of the experiment (Day 0) and every 5 days over the course of the treatment period. Tumor mass in each Group IV-VII mouse was measured starting on Day 3 after tumor cell injection. Measures of tumor radii (performed at right angles to one another) were taken every 3 d for a period of 30 days. Volume of the tumor mass was calculated using V=4/3 πr₁r²r₂, where r₁ and r₂ are the radii of the tumor (Majumdar, 1997). During this same period, blood from six mice/group was collected from the tail vain and total WBC (white blood cell) counts performed using a hemocytometer. A baseline value was also obtained on Day 0 from all mice and prior to injection of Group IV-VII mice with tumor cells.

At time of sacrifice on day 10 and day 15 post-tumor injection, 6 mice from each group were euthanized and their femurs collected to permit analyses of bone marrow samples. Blood was also collected for analysis (see below). Specifically, marrow cells were collected from both femurs and made into single cell suspensions and aliquots placed on glass slides (and stained with hematoxylin) for evaluation of non-specific α-esterase activity (using azodye coupling method; Bancroft and Cook, 1984). The remaining three mice in each group were maintained to monitor lifespan/tumor growth.

Effect of extract on serum TNFa, iNOS, IL-1β, IL-6, IL-2, IFNγ, GM-CSF, VEGF levels

Blood was collected from each animal on Days 15 and 30 and the corresponding serum assessed for tumor necrosis factor (TNF)-α, iNOS, interleukin (IL)-1β, IL-6, IL-2, interferon (IFN)-γ, GM-CSF, and VEGF levels using highly specific quantitative sandwich ELISA kits. The kits for evaluating mouse IL-1β, IL-6, IL-2, GM-CSF, VEGF, and IFNγ were procured from Koma BiotechTM (Seoul, Korea). Kits for measuring TNFα and iNOS were from USCN Life Sciences (Hubei, China). Limit of sensitivity of the IL-1β, IL-6, IL-2, TNFα, iNOS, VEGF, IFNγ, and GM-CSF kits were, respectively, 15, 5, 4, 3, 6.5, 23, 23 and 8 pg/ml.

Statistical analysis

All values are expressed as mean ± SD. For each endpoint, group means were compared using a one-way analysis of variance (ANOVA) followed by a Dunnett’s post-hoc test (Instat Version 3.0 software; Graphpad, San Diego, CA). A p-value < 0.05 was considered significant.

Results

Changes in MST and %ILS due to B. tomentosa extract

Mice injected with DAL cells (control) had an MST of 16.60 ± 1.03 days. This value was significantly increased to 25.33 ± 2.07 days due to the B. tomentosa extract treatment. These results are almost comparable to that of cyclophosphamide (CTX), the standard drug for which the MST was 29.83 ± 1.16 days. The % ILS of treated mice increased 52% compared to that of the DAL-bearing control hosts. In addition, the body weights of DAL tumor control mice increased 39% (relative to Group 1 mice controls); this value was reduced ≈17% due to the treatment with B. tomentosa (Table 1). These changes are important as host body weight is directly reflective of increases in DAL tumor volume in the peritoneal cavity.

Effect of extract on hemoglobin and WBC counts in DAL-bearing mice

The hemoglobin (Hb) content in the DAL control mice was decreased (10.22 ± 0.39 gm %) compared to that in normal mice (15.72 ± 0.40 gm %). Treatment with B. tomentosa at of 10 mg/kg. BW amplified the Hb content to moreover normal levels by Day 15 (15.78 ± 0.42 gm %). Total WBC counts in DAL control mice were increased (15.54 x 10³ cells/mm³) compared to those in with normal mice (11.0 x 10³ cells/mm³). Administration of the test extract led to reductions in the WBC counts on both Days 10 and 15 (respectively, 11.14 and 14.26 x 10³ cells/mm³) compared to those seen in the DAL control hosts (Figure 1).

Effect of extract on body weight changes due to DAL tumor and/or CTX treatment

Changes in host body weight during the experimental period (recorded before and every third day of experiment - up to Day 30) are shown in Figure 2. The results indicate CTX-treated mice had reduced body weight gains

Table 1. Effect of B.tomentosa on Mean Survival Time (MST), Percentage increase in Lifespan and Body weight

<table>
<thead>
<tr>
<th>Treatment design</th>
<th>MST (in days)</th>
<th>Percentage increase in lifespan</th>
<th>Bodyweight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>&gt;50</td>
<td>--</td>
<td>10.29</td>
</tr>
<tr>
<td>DLA Tumor Control</td>
<td>16.60 ±1.03</td>
<td>--</td>
<td>38.77</td>
</tr>
<tr>
<td>Tumor +, Methotrexate (3.5 mg/Kg/B.wt)</td>
<td>29.83 ±1.16**</td>
<td>79%</td>
<td>13.58</td>
</tr>
<tr>
<td>B.tomentosa (10mg/Kg/B.wt)</td>
<td>25.33 ±2.07**</td>
<td>52%</td>
<td>16.70</td>
</tr>
</tbody>
</table>

*The results are expressed as the mean of each group (n=6) and ** statistically significant different (p<0.01) from DLA tumor control animals.

Figure 1. Effect of B.tomentosa on Haemoglobin and WBC Count During DAL Bearing BALB/c Mice on 10th and 15th Days. Mice were injected with B. tomentosa extract (IP; 10 mg/kg. BW) daily for 10 day following initial IP injection of DAL cells (1.5 x 10⁶ DAL/mouse). Values shown are means (±SD); n=6/group and ** (P<0.01) Statistically Significant different from DAL tumor control animals.
compared to normal hosts, while DAL-injected mice had increasing weights due to tumor growth. In contrast, *B. tomentosa* extract-treated mice showed slower body weight gains relative to those of DAL-only mice. In mice treated with both CTX and *B. tomentosa* (in conjunction with DAL), body weight changes were on par with those in normal hosts.

**Effect of extract on bone marrow cellularity and α-esterase activity changes due to DAL tumor/CTX**

The effect of the test extract on bone marrow cellularity and α-esterase activity on Day 10 & 15 were shown in Figure 3. By Day 15, in non-tumor cell-injected mice, those that received CTX alone had an average of 38.2 ± 3.3 x 10⁵ cells/femur; this was significantly increased to 68.2 ± 8.3 x 10⁵ cells/femur by the co-administration of the extract. In tumor cell-injected mice, those that had the DAL had cell values of 64.0 ± 4.5 x 10⁵ cells/femur at both timepoints. This value was significantly reduced by CTX treatment (to 27.4 ± 3.5 x 10⁵ cells/femur; in mice with DAL that were treated with *B. tomentosa* extract only or with the CTX-extract co-treatment, the values increased to 79.0 ± 9.5] and 70.7 ± 7.8 x 10⁵ cells/femur, respectively. The trends of effect of the extract (alone or in combination with CTX) were paralleled in the measures of α-esterase activity (Figure 5).

**Effect of extract on tumor volume changes due to DAL tumor/CTX**

Effect of *Bauhinia tomentosa* on tumor volume during DAL induced solid tumor with CTX treatment results were shown in Figure 4. Tumor volume of DAL control animal was gradually increasing on 30th day this level was 2.52 ± 0.14 mm³ while CTX and *B. tomentosa* treated groups were significantly reduced to 0.88 ± 0.07 and 1.20 ± 0.10 mm³, respectively, on the same day. More over the combined treatment with *B. tomentosa* and CTX
increased evidently towards normal level. A reduction in the level of total WBC was seen in CTX treated control animals throughout the period of study. Whereas, WBC level in DAL solid tumor bearing group and DAL with *B. tomentosa* treated group shows gradually amplified and reach greater then a normal level (11.68 ±0.35x10^6^/mm^3^ and 12.35±0.46 x10^6^/mm^3^) respectively, but CTX treated along with DAL and *B. tomentosa* treated group the WBC level was reach nearby normal level at end of the experiment. Results were shown in Figure 5.

**Discussion**

Interest in alternative therapies using natural products for cancer treatment are increasing due to the wide range of toxic side effects of many currently-used chemotherapeutics (Jemal et al., 2009). Many plants extract and their active compounds have been studied in in vitro and in vivo cancer models (Newman and Cragg, 2012). Previous studies from our laboratory showed that *B. tomentosa* preparations appeared to impart various biological effects, such as acting as an immunomodulant, as well as imparting anti-oxidant, anti-inflammatory, and anti-tumor growth effect (in vitro only). (Kannan et al., 2010). The present study showed there was an anti-tumor effect induced by a *B. tomentosa* extract in vivo, in both an ascites and a solid tumor model. The preparation used here inhibited tumor/cancer growth in both models, as well as amplifications of several key hematologic parameters compared to values seen in hosts with the tumors but not receiving the extract.

 Normally, ascites tumor implantation induces local inflammation and increases in vascular permeability, cellular migration and, finally, progressive ascites fluid accumulation (Deepak et al., 2009). Here, the DAL control group had a shorter survival profile than that of hosts that also received the *B. tomentosa* extract (Figures 6 and 7). The patterns of changes in IL-2 and IFNγ levels differed from those of the other mediators, i.e., levels were lower in the tumor control hosts and then markedly elevated due to treatment with extract.

**Effect of extract on changes in serum inflammatory proteins due to DAL tumor/CTX**

Serum concentrations of select inflammatory mediators were measured by ELISA. The elevated level of serum TNFα, iNOS, IL-1β, IL-6, GM-CSF, and VEGF in tumor control mice, were reduced in hosts that also received the *B. tomentosa* extract (Figures 6 and 7). The patterns of changes in IL-2 and IFNγ levels differed from those of the other mediators, i.e., levels were lower in the tumor control hosts and then markedly elevated due to treatment with extract.

**Effect of extract on WBC level changes due to DAL tumor/CTX**

The mice which were treated with CTX has the total WBC count of 3.10 ±0.27 x10^6^/mm^3^ on 12th day and the animals treated with *B. tomentosa* the total WBC count were 23.437 ±0.37cells/mm^3^ on same day. The total WBC treated group resulted more reduction (0.68 ±0.06mm^3^) then other group of animals.
injected hosts, but also seemed to protect their bone marrow cells. It is known that CTX has a pro-oxidant character and generates oxidative stress; metabolites of CTX also bind to DNA and cause damage (chromosomal breaks, micronuclei formation) and cell death (Murata et al., 2004; Sakthivel et al., 2012). Here, administration of B. tomentosa extract in conjunction with CTX was found to enhance total WBC counts whereas these levels were drastically reduced in mice that received CTX alone. Bone marrow cellularity was also increased significantly in the co-treated hosts, indicating that extract appeared able to stimulate the hemato-poietic system. Moreover, there was increased presence of α-esterase marrow cells, suggesting to us that the extract could also enhance differentiation of stem cells. Whether the CTX-induced myelosuppression was reversed, inhibited, or both by the B. tomentosa extract remains to be more precisely defined.

In DAL control mice here, tumor volume was increased ≈8-times over a 30-d period, but only 4-times in animals treated with B. tomentosa; the effect was even greater in hosts that received extract-CTX co-treatment (only 2-times greater). These results suggested to us that the inhibitory effect related not only reduce the side effect caused by CTX but also with the systemic disturb. Similar results were obtained by Natesan et al. (2007) using Careya arborea methanolic extract.

The role of NO levels during tumor formation is regulated by interactions between endothelial cells in the tumor and infecting immune cells like macrophages and T-cells (Ruttimann, 2007). The Nitric Oxide synthase (iNOS) activity during tumor formation correlates positively with tumor grade (Kolb, 2000). Taken in that context, the results of the present study concerning NO and iNOS confirmed the anti-tumor effect of the B. tomentosa extract in the mouse hosts.

Immune cells have a broad impact on tumor initiation, growth, and progression; many of these effects are mediated by pro-inflammatory cytokines. Expression of these cytokines, i.e., interleukin (IL)-6, tumor necrosis factor (TNF)-α, IL-1β, and IL-2, are critical mediators during tumor progression involves regulation of transcription factors and nuclear factor (NF)-κB (Goldberg and Schwertfeger, 2010). Manusama et al. (1996) reported a synergistic anti-tumor effect with a combination of TNF-α and chemotherapeutics. One of the chemotherapeutic drug liposomal doxorubicin showed anti-tumor activity with presence of low dose of TNF-α in mice and rats. (Tenhagen et al., 2000). B. tomentosa treated group amplifications of TNF-α level may be helped to tumor suppression in extract treated group.

IL-6 is multifunctional immunomodulatory cytokines, which activate B and T lymphocytes produced by both normal and tumor cells and also serum concentration of IL-6 can used to predict progress of tumor cells (Cheng et al., 2014). IL-1β involved in the proliferation, differentiation and apoptosis of cells. Inflammatory hypersensitivity has been found to be the result of IL-1β activates COX-2. It has been implicated as a factor in tumor progression via the expression of metastatic and angiogenic genes and growth factors (Lewis et al., 2006). Granulocyte macrophage-colony stimulating factor (GM-CSF) has been shown to increase the immune response both in animal models and clinical trials. (Sakthivel and Guruvayoorappan, 2013) which was consistently induces dense CD4+ and CD8+ T-lymphocyte and plasma cell infiltrates, in metastatic lesions (Soiffer et al.,1998). The critical role of GM-CSF is not well characterized but clinical trials using GM-CSF-secreting tumors cells have been reported in patients with several tumor types (Small et al., 2007; Zarei et al., 2009). Vascular endothelial growth factor (VEGF) is a potent and specific angiogenic factor, was well known to be a key requirement for tumor growth. They activate endothelial cells to proliferate and migrate, subsequently resulting in new tube formation and blood flow (Chen et al., 2014). However, the result gained in this study indicates that B. tomentosa reduced the production of TNFα, iNOS, IL-1β, IL-6, GM-CSF, and VEGF levels clearly express the regulatory effect of this intermediates and tumor inhibition of B. tomentosa.

Interleukin (IL)-2 produced by T-cells (Malek, 2003) is considered a key growth and cell death factor for antigen-activated T lymphocytes IL-2 and IL-2 receptor deficient mice exhibit lethal autoimmunity (Preethi et al., 2010). Ouyang et al. (2006) conformed that IL-2 augmented activation of (TAM) would play the main role in induction of the MHC class I molecule through secretion of IFNγ, and would contribute to the IFNγ-mediated apoptosis induction in tumor cells. IFNγ, an important immunoregulatory molecule against tumor cells, appears capable of driving novel cellular and molecular inflammatory mechanisms that may underline tumor initiation, immunoevasion, survival, and IFN-γ could inhibit angiogenesis and cell proliferation (Blankenstein and Qin, 2003).

In conclusion, the potential anti-cancer effect of B. tomentosa was investigated and it was found that a B. tomentosa extract exhibited a strong inhibitory effect on the proliferation of both solid and ascites tumor cells. The results of this experimental study suggested that B. tomentosa contains some active constituents that could be useful as anti-cancer drugs. The activity of the extract could be attributed, in part, to effects on tumor cell proliferation, immune system activation, and/or a blocking (or induction) of key immune system regulatory products (including TNFα, IL-2, IL-6, IL-1β, GM-CSF, or IFNγ) that can impact on tumor cells during proliferation, cell cycle regulation, signal transduction, or even the motility and invasiveness of the cancer cells.

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