RESEARCH COMMUNICATION

**Andrographis paniculata** Downregulates Proinflammatory Cytokine Production and Augments Cell Mediated Immune Response in Metastatic Tumor-Bearing Mice

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**Abstract**

Effects of *Andrographis paniculata* extract and its major component, andrographolide, on cell-mediated immune responses in metastatic tumor bearing animals were studied. NK cell mediated target cell lysis was enhanced by the administration of *Andrographis paniculata* extract (45.0% cell lysis) and andrographolide (40.2% cell lysis) on the 5th day after tumor induction when compared to untreated metastatic tumor bearing animals in which maximum target cell lysis was observed on 11th day (11.4%). Antibody dependent cell-mediated cytotoxicity (ADCC) was also enhanced by treatment with the extract (42.0% cell lysis) and andrographolide (40.2%) in comparison with the untreated case (11.0%). Similarly, the extract (25%) and andrographolide (22%) showed higher ACC activity than the control (14%) and treatment of extract and andrographolide resulted in significant increase in serum IL-2 and TIMP-1 levels. Furthermore, the levels of proinflammatory cytokines such as IL-1β, IL-6, GM-CSF and TNF-α were effectively reduced by the administration of extract and andrographolide in metastatic tumor bearing animals.

**Keywords:** Cytokines - metastasis - NK cells - andrographolide - cell mediated immunity

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**Introduction**

Despite intense scrutiny, cancer remains a formidable disease with limited therapeutic options, especially in the context of metastatic disease (Fisher, 2005). Metastasis, formation of secondary tumors is a turning point in tumourogenesis and consists of complex series of parallel, yet intertwined events (Bogenrieder, 2003). Several molecules and signals in the host system especially in the tumor microenvironment, which include, inflammatory cells, cytokines etc. forms critical mediators of neoplastic progression and metastasis (Schwertfeger et al., 2006).

Cell mediated immune system is responsible for early detection and elimination of tumor cells. Cytotoxic T lymphocytes (CTL), natural killer cell (NK cell), NKT cell, dendritic cells and macrophages are mainly involved in this process (Smyth et al., 2001). Although most tumors are immunogenic, the immunity they evoke is either too weak to reject a rapidly growing tumors or they suppress the host immune system (Old et al., 1962; Naor, 1979). In fact a state of persistent chronic inflammation associated with cancer may attenuate T cell function, which is essential for immunosurveillance and cancer cell destruction (Roung-Fa Wang, 2006). A healthy immune system is necessary for control of malignant disease (Whiteside, 2006). Hence, augmentation of host natural defence machinery becomes one of the effective therapeutic treatment approaches against primary as well as secondary neoplasm.

Plants and plant-derived compounds find very much importance in various diseases including cancer. *Andrographis paniculata* is a herb found throughout India and other Asian countries. Preliminary studies in animals suggest that this plant may offer benefits for preventing heart diseases (Zhao and Fang, 1991). It may help to protect the liver from toxic injury, perhaps more effective than the more famous liver-protective herb milk thistle (Visen et al., 1993). It also possesses antidiabetic property (Zhang and Tan, 2000). Andrographolide, primary active ingredient in *Andrographis paniculata*, is a bitter diterpenoid lactone is used for the treatment of common cold and it possess anti-inflammatory (Shen et al., 2002) and antihepatic activity (Kapil et al., 1993). It was found to be cytotoxic to a variety of transformed cell lines (Kumar et al., 2004).

In the present study we investigated the effect of *Andrographis paniculata* extract and its major component andrographolide on cell mediated immune response in metastatic tumor bearing animals.

**Materials and Methods**

**Animals**

C57BL/6 mice (6-8 weeks) weighing 20-25g

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were purchased from National Institute of Nutrition, Hyderabad, India. Animals were housed in well-ventilated cages and kept in air-controlled room. They were fed with normal mouse chow (Sai Durga Feeds, India) and water ad libitum. All animal experiments were performed according to the rules and regulations of Animal Ethics Committee, Govt. of India.

**Cell lines**

B16-F-10 melanoma highly metastatic cells and K-562, a natural killer cell sensitive human erythroleukemic cell lines were purchased from National Centre for Cell Sciences, Pune, India. B16-F10 cells were maintained in DMEM and K-562 cells were maintained in RPMI-1640 supplemented with 10% FCS (Biological Industries, Israel) and antibiotics.

**ELISA kit**

Highly specific ELISA Kits for TNF-α, IL-1β, GM-CSF, IL-6 and IL-2 were purchased from Pierce Biotechnology, USA and TIMP-1 ELISA kit was purchased from R&D system, USA.

**Chemicals**

Sodium chromate (Na₂51CrO₄) was purchased from Board of Radiation and Isotope Technology, Mumbai, India. Andrographolide was purchased from Natural Remedies, Bangalore, India.

Fresh sheep red blood cells (SRBC) were collected in Alsever’s solution from local slaughter house. Rabbit serum was collected from Amala Cancer Research Centre. All other reagents were of analytical reagent grade.

**Preparation of the plant extract**

Air dried whole plant was powdered and extracted using 70% ethanol by stirring overnight at 4°C. Supernatant was collected after centrifuging at 10,000 rpm at 4°C for 10 min. Ethanol was evaporated. The yield of the extract was 14%. Phytochemical analysis of the extract showed the presence of terpenoids and flavanoids.

**Isolation of andrographolide**

Andrographolide (see Figure 1) was extracted from the dried plant powder according to method of Handa and Sharma (Handa and Sharma, 1990). Briefly, the whole plant material was dried, powdered and extracted at room temperature successively with 4x40 liter petroleum ether (60-80°C), Chloroform and methanol in soxhlet extraction assembly. The methanolic extract was concentrated and activated charcoal (400g) was added to it. After 24 h, charcoal was filtered off and the filtrate was concentrated under reduced pressure and left overnight for crystallization. The crystals were collected by filtration and purified by recrystallization. The isolated andrographolide was compared with an authentic sample by thin layer chromatography and mass spectra.

**Administration of Andrographis paniculata extract and andrographolide**

For the animal administration, Andrographis paniculata extract and andrographolide were dissolved in minimum volume of ethanol and resuspended in 1% gum acacia and was given intraperitoneally (i.p) at a concentration of 10mg/dose/animal and 500 µg/dose/animal, respectively.

**Determination of natural killer cell (NK cell) activity, antibody dependent cell mediated cytotoxicity (ADCC) and antibody dependent complement-mediated cytotoxicity (ACC) in metastatic tumor bearing animals**

C57BL/6 mice grouped into three (12 nos./group). All the animals were injected with B16 F-10 cells (10⁶ cells/animal) intravenously through tail vein. Group I animals were kept as untreated metastatic tumor bearing control. Animals in group II animals and III were treated with 5 doses of extract (10mg/dose/animal) and andrographolide (500µg/dose/animal) respectively. Animals were sacrificed at different time periods after tumor induction. Blood was collected by heart puncture. Spleen was excised and processed to get single cell suspension and was used as effector cells to determine NK-cell activity and ADCC by 51Cr-release assay. Chromium labelled K-562 cells were used as the target cells for determining NK activity and Chromium labelled SRBC as the target cells for ADCC. Antibody raised against SRBC in rabbit was used as the source antibody in ADCC assay.

Serum was separated from blood, heat inactivated at 56°C and used as a source of antibody for ACC.

**Natural killer cell (NK cell) mediated cytotoxicity in metastatic tumor bearing animals**

NK cell activity was determined using NK cell sensitive K-562 cell line by standard 51Cr release assay (Kim et al., 1980). 51Cr labelled target cells (K-562) and spleen cells from extract or andrographolide treated mice were mixed to get an effector target ratio of 100:1, in 96 well titre plate. Final volume was adjusted to 200µl with RPMI-1640 supplemented with 10% FCS and incubated at 37°C in 5% CO₂ atmosphere for 4h. After incubation, the plates were centrifuged at 1500rpm for 15 minutes, supernatant was collected and radioactivity was measured in a γ-ray counter. Total and spontaneous release was determined by incubating radiolabelled target cells in the presence and absence of 1N HCl respectively. All the tests were done in triplicate and percentage target cell lysis was calculated using the formula:

![Figure 1. Chemical Structure of Andrographolide](image-url)
% target cell lysis =
Experimental release - Spontaneous release X 100/
Total release - Spontaneous release

**Determination of antibody-dependent cellular cytotoxicity in metastatic tumor bearing animals**

Antibody-dependent cellular cytotoxicity (ADCC) was determined by chromium release assay (Kim et al., 1980) using chromium labelled sheep red blood cells (SRBC) as the target cells. Spleen cells from the metastatic tumor bearing control and treated animals (extract as well as andrographolide) as effector cells. The effector and target cells were added in 96 well titre plate at a ratio of 100: 1 (effector to target ratio) and incubated in the presence on anti SRBC Ab for 4h at 37°C. The activity of chromium released into the culture supernatant was determined and percentage of target cell lysis was calculated as explained above.

**Determination Antibody dependent complement mediated cytotoxicity (ACC) in metastatic tumor bearing animals**

Serum collected from all the experimental animals was diluted in RPMI-1640 to get 1:1 dilutions. To 100μl of diluted serum sample, 100μl of B16F10 melanoma cells (1x10⁶ cells) and 50μl of complement were added and final volume was made to 2 ml with complete RPMI 1640 medium and incubated at 37°C for 3h. Serum sample with B16F-10 cells alone, B16 F-10 cells with complement, B16 F-10 cells with antibody were kept as controls. The cytotoxicity was assessed by trypan blue exclusion method (Gupta and Bhattacharya, 1983).

**Determination of the effect of Andrographis paniculata extract and andrographolide on cytokine production by metastatic tumour bearing animals**

B16F-10 melanoma cells (10⁶ cells) were injected to each animal via lateral tail vein. The animals were divided into three group comprising six animals in each group. Group I animals were kept as untreated metastatic tumor bearing control. Groups II and III were received 5 consecutive doses of extract (10mg/dose/animal) and andrographolide (500µg/dose/animal) respectively. Blood was collected by tail bleeding on 7th and 21st day after tumor challenge and serum was separated and used for the estimation of TIMP-1 level and cytokines such as IL-1β, IL-6, GM-CSF, TNF-α, IL-2 using ELISA kits as per manufacture’s protocol.

**Statistical analysis**

All data are expressed as mean ± Standard Deviation (SD). The normal group was compared with tumor bearing untreated control group by student’s t-test. The tumor bearing untreated control group was compared with extract or andrographolide treated tumor bearing group by one-way ANOVA followed by Bonferonni’s multiple comparison test.

**Results and Discussion**

**Effect of Andrographis paniculata extract and andrographolide on NK cell activity in metastatic tumour bearing animals**

As shown in Figure 2 treatment with extract as well as andrographolide significantly enhanced the NK cell activity and it was observed much earlier compared to untreated metastatic tumor bearing control animals. Maximum NK cell mediated target cell lysis was observed on 5th day by the administration of extract (45.0% cell lysis) and andrographolide (40.2% cell lysis). In metastatic tumor bearing control the maximum NK cell activity was observed only on day 11 and it was 11.4% cell lysis, while NK cell mediated targeted cell lysis in normal mice was 1.14%. Among various arms of immunity, antigen non specific immunity, mediated by NK cells, macrophages, dendritic cells represent first line of defence against the developing tumors and play an important role in immune surveillance against cancer cell (Karin E de et al., 2006). Circulating tumour cells are particularly sensitive to lysis by natural killer cells or monocytes (Whiteside and Herberman, 1995). Our study showed that treatment with extract and andrographolide significantly enhanced the NK cell mediated target cell lysis indicate that they could activate NK cells in metastatic tumor bearing animals and thereby prevent secondary growth of B16 F-10 melanoma cells.

**Effect of Andrographis paniculata extract and andrographolide on ADCC in metastatic tumor bearing animals**

ADCC was also enhanced by the administration of extract as well as andrographolide in metastatic tumor bearing animals compared to untreated metastatic tumor bearing control. Both extract and andrographolide treated animals showed maximum ADCC on 11th day with peak target cell lysis 42.0% and 40.2% respectively, where as in tumor bearing untreated control animals peak NK activity was observed only on 15th day (11.0% cell lysis) (Figure 3). In normal animal the ADCC activity was only 0.96%. In addition to direct lysis of target cells, various effector cells such as neutrophils, monocytes, macrophages, dendritic cells and NK cells would also be able to mediate antibody dependent cellular cytotoxicity (ADCC), in which antibody coated target cells are killed by these cells.
Figure 3. Effect of Andrographis paniculata Extract and Andrographolide on ADCC in Metastatic Tumor-bearing Animals. All the animals were injected with B16 F-10 cells (10⁶ cells/animal) intravenously through tail vein. Group I animals were kept as untreated metastatic tumor bearing control. Animals in group II and III were treated with 5 doses of extract (10mg/dose/animal) and andrographolide (500µg/dose/animal) respectively to determine ADCC by 51Cr-release assay.

Figure 4. Effect of Andrographis paniculata Extract and Andrographolide on ACC in Metastatic Tumor-bearing Animals. All the animals were injected with B16 F-10 cells (10⁶ cells/animal) intravenously through tail vein. Group I animals were kept as untreated metastatic tumor bearing control. Animals in group II and III were treated with 5 doses of extract (10mg/dose/animal) and andrographolide (500µg/dose/animal) respectively. Blood was collected serum separated, heat inactivated at 56°C and used as a source of antibody for ACC effector cells (Mellsledt, 2003). Many investigators have reported ADCC mediated by PMN in colorectal and breast cancer (Tamanorri, et al., 2002). Administration of extract and andrographolide increased ADCC in metastatic tumor bearing animals when compared to untreated metastatic tumor bearing animals suggest that extract and andrographolide stimulate both cell mediated and humoral responses to eliminate the tumor and block metastasis.

Effect of Andrographis paniculata extract and andrographolide on ACC in metastatic tumor bearing animals

Significant enhancement in ACC was also observed in extract and andrographolide treated metastatic tumor bearing animals. Maximum activity was observed in extract (25% cell lysis) and andrographolide (22% cell lysis) treated animals on day 15 while in untreated metastatic tumor bearing control animals ACC activity was 14% cell lysis which was on day 19 (Figure 4). Complement as an essential component of the immune system is of substantial relevance in tumor cells destruction. The complement proteins under normal condition exist in inactive proenzyme. Activation of complement system by antibody may trigger cascade of reactions finally terminate in the death of target cells (Goldsby et al., 2002). In the presence of complement, serum from extract and andrographolide treated metastatic bearing animals were shown to be more cytotoxic to B16 F-10 melanoma cells compared to serum from untreated metastatic tumor bearing control animals suggest that extract and andrographolide activate the humoral immune system to produce specific antibody against the developing tumor thus mediate target cell death through ACC, which in turn inhibit tumor development.

Effect of Andrographis paniculata extract and andrographolide on various cytokines (IL-1β, IL-6, GM-CSF, TNF-α IL-2) and TIMP-1 production in metastatic tumor bearing animals

As shown in Figure 5 on 7th day after tumor induction, metastatic tumor bearing control animals showed on elevated level of IL-1β (32.1±1.05pg/ml) compared to normal level (17.8±2.6 pg/ml). Administration of extract (26.0±0.95 pg/ml) and andrographolide (27.6±1.6905pg/ml) reduced this elevated level on the same day. On 21st day the level of IL-1β in metastatic tumor bearing control animals was again elevated to 53.6±1.31 pg/ml while it was significantly lowered in the extract (19.89±0.79 pg/ml) as well as andrographolide (23.9±2.35 pg/ml) treated group of animals. Tumor progression has been reported to be invariably associated with alterations in cytokine balance, which plays a central role in manifesting unhindered tumor growth along with suppression of hosts antitumor immune responses (Lauero et al., 2002). Developing tumors induce a local and systemic chronic inflammatory environment associated with enhanced tumor growth and metastasis through production of various cytokines (Baniyash, 2006).

Normal level of IL-6 is 35.9±3.6pg/ml which was found to be drastically elevated in metastatic tumor bearing control animals on 7th day after tumor induction.
Metastatic Tumor-bearing Animals. Animals were divided into three group comprising six animals in each group. Group I animals were kept as untreated metastatic tumor bearing control. Group II and III were received 5 consecutive doses of extract (10mg/dose/animal) and andrographolide (500µg/dose/animal) respectively. Blood was collected on 7th and 21st day after tumor challenge and serum was separated and used for the estimation of IL-6 level.

Figure 6. Effect of Andrographis paniculata Extract and Andrographolide on Serum IL-6 Level in Metastatic Tumor-bearing Animals. Animals were divided into three group comprising six animals in each group. Group I animals were kept as untreated metastatic tumor bearing control. Group II and III were received 5 consecutive doses of extract (10mg/dose/animal) and andrographolide (500µg/dose/animal) respectively. Blood was collected on 7th and 21st day after tumor challenge and serum was separated and used for the estimation of IL-6 level.

Figure 7. Effect of Andrographis paniculata Extract and Andrographolide on Serum GM-CSF Level in Metastatic Tumor-bearing Animals. Animals were divided into three group comprising six animals in each group. Group I animals were kept as untreated metastatic tumor bearing control. Group II and III were received 5 consecutive doses of extract (10mg/dose/animal) and andrographolide (500µg/dose/animal) respectively. Blood was collected on 7th and 21st day after tumor challenge and serum was separated and used for the estimation of GM-CSF level.

(335.1±3.65 pg/ml). Administration of extract (68.3±3.77 pg/ml) and andrographolide (72.1±1.66 pg/ml) could reduce this elevated level on the same day. Serum IL-6 in control animals was again elevated as tumor progress, to 580.28±16.60 pg/ml on 21st day. Extract (235.9±12.21 pg/ml) and andrographolide (257.4±4.39 pg/ml) administered animals showed significantly reduced level of IL-6 on the same day (Figure 6). IL-6 a proinflammatory cytokine has been suggested to be a mediator of morbidity and mortality in patients with metastatic disease (Drachenberg et al., 1999). It affects the differentiation of cells myeloid lineage, through the activation of the transcription factor STAT which exerts a negative immune regulatory function during tumor development (Trikha et al., 2003). We found that serum IL-6 level was significantly decreased after administration of extract and andrographolide when compared to untreated tumor bearing control animals and may help to inhibit growth and metastasis of developing tumors.

GM-CSF level in serum of metastatic tumor bearing control animals were highly elevated to 53.01±2.56 pg/ml on 7th day after tumour challenge and was reduced significantly by extract (24.6±2.26 pg/ml) and andrographolide (25.9±2.39 pg/ml). On 21st day the level of GM-CSF in control animal was found to be 24.5±0.75 pg/ml and was decreased to 11.7±1.67 pg/ml and 14.2±1.38 pg/ml by the administration of extract and andrographolide respectively. Normal level of serum GM-CSF is 18±3.1 pg/ml (Figure 7). Takeda et al. (1991) reported that granulocyte monocyte colony stimulating factor (GM-CSF) secretion correlated with the capacity to metastasize various transplanteable mouse tumors when injected subcutaneously. Here, we reported that administration of extract and andrographolide could decrease the level of GM-CSF in metastasis tumor bearing animal and this result provide another evidence for the reduction in the inflammatory state which inturn inhibit the tumor progression.

Similarly, serum TNF-α level was elevated in metastatic tumor bearing control animals (262.1±8.42 pg/ml) on 7th day after tumor induction compared to normal level (20±3.2 pg/ml) (Figure 8). This elevated level was effectively lowered by the administration of extract (145.5±5.30) and andrographolide (153.3±5.53) on the same day. In control animals TNF-α level was further elevated on 21st day after tumor induction (630.8±9.54) and was significantly reduced after treatment with extract and andrographolide to 65.4±3.3 pg/ml and 74.8±3.42 pg/ml. TNF-α, a potent proinflammatory cytokine, promotes metastasis by inducing expression of MMPs and intergrins (Waterson, 2004). The reduced level of TNF-α in the metastatic tumor bearing control animals by the administration of extract and andrographolide directly related to its antitumetic activity.

IL-2 level in normal animal is 22.2±2.6 pg/ml. Decreased level of IL-2 on 7th day after tumor induction in metastatic control animals (18.7±1.31 pg/ml) was found to be significantly elevated by the administration of extract (35.5±2.65 pg/ml) and andrographolide (30.2±3.2 pg/ml) on 7th day after tumor induction. The IL-2 level was again increased in extract (51.5±1.31 pg/ml) and andrographolide (48.7±1.26 pg/ml) treated animals on 21st day of tumor induction while the control group
level of IL-2 was further lowered to 9.77±1.09 pg/ml on the same time point (Table 1). Interleukin -2, a T cell growth factor as well as the main stimulator of NK cells augment host’s cell mediated antitumor immune response (Nishimura et al., 1999; Rodriguez et al., 2005). The above result shows that extract and andrographolide effectively stimulate the NK cell and T cell mediated immune responses in metastatic tumor bearing mice by enhancing the production of IL-2 and thus eliminate the cancer cells.

There was a significant enhancement in the TIMP-1 level by the administration of extract and andrographolide compared to normal as well as untreated metastatic tumor bearing animals (Table 2). TIMP-1 level in metastatic tumor bearing control animals was 553.96±21.41 pg/ml which was found to be increased by the administration of extract (640.20±24.09 pg/ml) and andrographolide (671.8±35.8 pg/ml).

Table 1. Effect of Andrographis Paniculata Extract and Andrographolide on IL-2 Profile in Metastatic Tumor Bearing Mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>IL-2 (pg/ml)</th>
<th>Day 7</th>
<th>Day 8</th>
</tr>
</thead>
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<tr>
<td>Normal</td>
<td>22.2±2.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>18.67±1.3</td>
<td>9.77±1.09</td>
<td></td>
</tr>
<tr>
<td>Extract treated</td>
<td>35.46±2.65</td>
<td>51.51±1.3</td>
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<tr>
<td>Andrographolide treated</td>
<td>30.20±0.32</td>
<td>48.71±1.26</td>
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</tr>
</tbody>
</table>

Table 2. Effect of Andrographis Paniculata Extract and Andrographolide on TIMP-1 Profile in Metastatic Tumor Bearing Mice

<table>
<thead>
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<th>Treatment</th>
<th>TIMP-1 (pg/ml)</th>
<th>Day 7</th>
<th>Day 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>596±43</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>553.96±21.41</td>
<td>493.61±18.69</td>
<td></td>
</tr>
<tr>
<td>Extract treated</td>
<td>640.20±24.09</td>
<td>591.71±33.78</td>
<td></td>
</tr>
<tr>
<td>Andrographolide treated</td>
<td>671.8±35.8</td>
<td>654.29±23.67</td>
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</tbody>
</table>

B16 F-10 melanoma cells (10/50µl) were injected to mice via lateral tail vein. Blood was collected from the metastasis-induced animals at the specified time point after tumor challenge. Serum was separated and levels of IL-2 was estimated by ELISA method; All the values are mean ±SD *** P<0.001

correlate with their role in stimulation of erythropoiesis and metastasis of B16 F-10 cells in vivo.

Summarising, the results of the present study suggest that Andrographis paniculata extract and its major component andrographolide could activate NK cells and ADCC and ACC in metastatic tumor bearing animal that may directly contribute towards the elimination of highly metastatic cell line, B16 F-10 in animal models. In this study andrographolide and the crude extract of Andrographis paniculata showed more or less similar results, suggest that andrographolide, present in the crude extract is the responsible constituent for the augmentation of cell mediated immune responses and inhibition of proinflammatory cytokine production.

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Andrographis paniculata Suppression of Cytokine Production and Stimulation of Cell-Mediated Immunity


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