Immunomodulatory activity of aqueous extract of *Actinidia macrospmera*

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*Actinidia macrospmera* (AM) is a medicinal plant in China and has been well known for its activities against cancers, especially of lung, liver and digestive system. The immunomodulatory effects of AM aqueous extract were examined using S180-bearing mice. Young adult (20 ± 2 g) ICR mice inoculated with S180 cells were divided randomly into six groups: S180-bearing control group, normal control group, positive control group (Ginseng Radix Rubra 2000 mg/kg) and 50, 100, 250 mg/kg AM treatment groups. Each group consisted of ten mice. Body and tumor weights were obtained after 12 consecutive days, and their humoral, cellular and nonspecific immune functions were also determined by relative assays. The results showed that the aqueous extract of AM was lack of significant inhibit on transplantable sarcoma S180, with a inhibit rate of 1.5%–14.8% (dose at 250 mg/kg was the best), but significantly increased the overall immune functions (especially at 100 and 250 mg/kg). The immunomodulatory effect was dose-dependent in a nonlinear fashion with the optimal dose of 100 mg/kg. The AM-induced antitumor effects were at least partially indirect and were associated with the modulation of immune functions.

Key Words: antitumor, immunomodulator, Sarcoma-180 (S180), *Actinidia macrospmera*

**Introduction**

The great majority of chemicals identified as cytotoxic to cancer cells are generally also toxic to normal cells. Nevertheless, the potentiation of host defense mechanisms has been recognized as a possible means of inhibiting tumor growth without harming the host. Therefore, searching for immunomodulatory materials from natural herbs and characterizing the immune enhancement effects may have great potential in cancer treatment, based on a combination of time honoured traditional usage and ongoing scientific research.

*Actinidia* species, commonly known as Kiwifruit, belong to the family *Actinidiaceae* and are distributed throughout the world, especially in eastern Asia. Traditionally they have been used to treat different cancers, including those of the digestive system and mammary gland. Because of a broad spectrum of pharmacological and biological properties of *Actinidia* as health food and folk medicine, there is a renewed interest in its chemical compositions and biological activities. A number of bioactive constituents have been reported, including polysaccharides, alkaloids, saponins, and organic acid. Some studies showed the kiwifruit juice inhibited the growth of cancer cells. The kiwifruit ethanolic extract exhibited a high of 51% inhibition by Ames’ test against the mutagenicity of N-nitrosodibutyramine (NDBA). Moreover, extracts from some species of kiwifruits inhibited the growth of cultured human hepatoma cell (BEL-7404) and Ehrlich ascites carcinoma and HeLa cells, and reduced the nitrosation reactions (which were important in cancer induction) by efficiently nitrile scavenging activity of ascorbic acid present in extracts.

*Actinidia macrospmera* (shorten as AM), a naturally wild kiwifruit and endemic to eastern China, is popularly called ‘Cat Ginseng’ because of the function attracting cats to use the plant as a stimulant and a healer for wounds. AM has been used as a herb medicine since the 1960s, and listed in TCM (Traditional Chinese Medicine) standards of Zhejiang Province. It has been extensively employed to treat various ailments like leprosy, abscess, rheumatism, arthritis inflammation, jaundice and abnormal leucorrhea etc. It has also been reported that AM was useful for the treatment of cancers, especially those of lung, liver and digestive system. The injection isolated from the plant had in vitro antitumor efficacy to there liver cancer cell lines (H22, CBRH-7919 and SMMC-7721). The active fraction from AM also showed the activity against the transplanted H22 in mouse by use of influencing cell cycle (arrest at G₀-G₁ phase and decrease at S phase) and inducing apoptosis. However, information regarding its active component and biological activities remains unclear so far.

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In the present study, we focused on the antitumor and immunomodulatory effects of the aqueous extract from AM on the S180 ascites tumor in mice, a widely recognized immunomodulator-sensitive allogeneic tumor mode for detecting potential agents preventing the recurrence of cancer diseases, and analyzed its involvement in the activation of cellular, humoral and nonspecific immunities.

Materials and methods

**Plant extract**

AM was obtained from local market in Zhejiang Province, China. The Lab of Plant Systematic Evolution and Biodiversity achieved the botanical identification. Voucher specimens were deposited in the Zhejiang University herbarium. The material was powdered and its aqueous extract was prepared using double distilled water. The homogenate was centrifuged at 2000 rpm for 10 min to remove the particles. The supernatant was then concentrated to dryness with a percentage yield of 3.89% (w/w) in a rotary evaporator (40°C under vacuum) and the resulting viscous mass was suspended in normal saline for the experiment. The doses were calculated as dry weight of materials (mg) used to prepare 1 ml extract.

**Experimental animals**

Young adult (20 ± 2 g) ICR mice (male and female in half) were provided by the Animal Center, Academe of Chinese Traditional Medicine, Zhejiang, China. All mice were raised in a 12/12 hours light-dark cycle room with controlled temperature (21 ± 2°C) and humidity (10%), and fed on standard rodent chow and water ad libitum.

**Effect of AM extract on the tumor of S180-bearing mice**

The effect of AM extract on tumor growth was estimated by evaluating body weight, tumor weight, and percentage of tumor inhibition. S180 tumor cell line was originally obtained from Shanghai Institute of Materia Medica, Chinese Academy of Sciences, and maintained as the ascites form by serial passages intraperitoneally in ICR mice. For solid tumor development, 0.2 mL of S180 cell suspension (2×10^7 cells/mL) was inoculated subcutaneously into right armpits of mice under sterile condition. The mice were divided into six random groups (10 in each): S180-bearing control, normal control, Ginseng Radix Rubra (GRR, 2000 mg/kg body weight), and three AM treatments (50, 100, 250 mg/kg body weight). Test doses were decided on the basis of findings from preliminary experiments. Body weight of animals was recorded before the experiment. GRR and AM extract were administrated p.o. daily for 12 days. Normal control and S180-bearing control groups received the same volume of normal saline. On the 13th day, all animals were executed. Their body and tumor weights were obtained and documented.

**Assessment of humoral immune function: Quantitative hemolysis of sheep red blood cells (QHS) assay**

The mice were injected i.p. with 0.2 mL of 3% 5 (v/v) sheep red blood cells (SRBC) prepared in normal saline on the 8th day of the experiment. QHS assay was performed in those animals following the immunization. Eyeballs were removed and single cell suspensions of 1×10^7/mL were prepared in phosphate buffer solution (PBS). A total of 1.0 mL of 0.4% SRBC and 1.0 mL of 10% guinea pig serum were mixed with cell suspension and incubated for 1 h at 37°C. After a 3 min centrifugation at 3000 rpm, the absorbance of the supernatant was measured at 413 nm using a spectrophotometer.

**Assessment of cellular immune function**

For the assessment of cellular immune function, lymphocyte proliferation and NK cell cytotoxicity tests were performed. After the experiment was completed, their spleens were aseptically removed, and filtered over a double layer of stainless-steel mesh to obtain single cell suspension. After these washes in Hanks' balanced salt solution, the spleen cells were finally suspended in 10% FCS RPMI 1640 media supplemented with benzylpenicillin 100 U/mL, streptomycin 100 μg/mL. The cell number was adjusted to 3×10^6 cells/mL of culture media for subsequent experiments.

**a) Measurement of lymphocyte proliferation**

For the splenocyte proliferation assay, the spleen cell suspension was added to microplate wells with 5 μg/mL of concanavalin A (ConA, from Canavalia ensiformis Type III, Sigma) and a polyclonal T cell mitogen. The microplates were cultured at 37°C for 72 h in the humidified 5% CO2 incubator. At 72 h, 1μCi/well of 3H-TdR (thymidine, [methyl-3H]) was added to each well. The cells were harvested 16 h later and the radioactivity incorporated was counted using a liquid scintillation counter.

**b) Evaluation of NK cell cytotoxicity**

The splenocytes prepared as described above were used as effector cells. YAC-1 cells, mice lymphoma sensitive to NK cells were used as target cells. Effector and target cells resuspended in RPMI-1640 medium supplemented with 3% heat-inactivated fetal bovine serum were added to each well of a 96-well U-bottom microculture plate in triplicate to obtain an effector/target (E/T) ratio of 50:1; and incubated at 37°C in the humidified 5% CO2 incubator for 8 h. After centrifugation, the culture supernatants were admixed with lactate dehydrogenase (LDH) solution (100μl/well) and the amount of released LDH was determined. The OD value of each well was measured at 490 nm using a spectrophotometer. The percentage of cytotoxicity generated by NK cells was calculated according to the following formula:

$$\text{Cytotoxicity (\%) } = \frac{\text{OD}_{	ext{tsr}} - \text{OD}_{\text{tsrtmr}} - \text{OD}_{\text{tsr}}}{\text{OD}_{\text{tsrtmr}} - \text{OD}_{\text{tsr}}} \times 100$$

Where OD_{tsr} (OD_{experimental release}) was the LDH release from co-cultures at an E/T ratio of 50:1; OD_{tsr} (OD_{target spontaneous release}) and OD_{tsrtmr} (OD_{target spontaneous release}) were spontaneous LDH releases from effector and target cells incubated with medium alone, respectively; and OD_{tsrtmr} (OD_{target maximum release}) was the maximum release from target cells lysed with the lysis solution.

**Assessment of nonspecific immune function: Phagocytic activity of macrophage**

Phagocytic activity of macrophages was used to assess the nonspecific immune function. The mice were injected i.p. with 0.5 mL 5% cock red blood cells (CRBC) 10 h prior to the last dose. On the 13th day of the experiment,
Effects of AM extract on humoral immune function bearing mice

Effects of AM extract on immune functions of S180-bearing mice.

Complete regression of tumor was not observed in any treatment appeared to show more effective inhibition. AM extract was 50, 100 and 250 mg/kg. Higher concentrations with water or any of the three AM aqueous extracts compared with S180 control group. The inhibit rate was 1.5%, but showed lower tumor growth at a certain extent compared with control group. All AM treatments could markedly increase the antibody secreted by spleen cells in mice. The effect of 100 mg/kg dose was the best and it could restore humoral immunity in S180-bearing mice close to Ginseng Radix Rubra (GRR) 2000 mg/kg, a positive control well known as a typical immunostimulator.

Statistical analysis
Data were expressed as the mean ± standard deviation (S.D.) in tables. Statistical analyses were carried out using the analysis of variance (ANOVA) and post-hoc tests for multiple comparisons. Differences were considered statistically significant at \( p<0.05 \).

Results

Effects of AM extract on body and tumor weights of S180-bearing mice
All the tumor-injected mice survived following the treatment with water or any of the three AM aqueous extracts until they were sacrificed for analysis of immunological features. When the tumor masses were removed, we observed that the animals treated with AM had no significant change in body and tumor weight (Table 1, \( p>0.05 \)), but showed lower tumor growth at a certain extent compared with S180 control group. The inhibit rate was 1.5%, 5.3% and 14.8%, respectively, when the concentration of AM extract was 50, 100 and 250 mg/kg. Higher concentrations appeared to show more effective inhibition. Complete regression of tumor was not observed in any group.

Effects of AM extract on immune functions of S180-bearing mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Concentration (mg/kg)</th>
<th>Body weight (g) Begin</th>
<th>Tumor weight (g)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>–</td>
<td>20.4 ± 1.22</td>
<td>28.5 ± 4.77</td>
<td>–</td>
</tr>
<tr>
<td>S180 control</td>
<td>–</td>
<td>20.5 ± 1.41</td>
<td>27.6 ± 3.50</td>
<td>4.15 ± 1.18</td>
</tr>
<tr>
<td>GRR a</td>
<td>2000</td>
<td>20.9 ± 0.98</td>
<td>28.1 ± 2.98</td>
<td>3.85 ± 0.75</td>
</tr>
<tr>
<td>AM extract b</td>
<td>100</td>
<td>20.1 ± 1.05</td>
<td>28.0 ± 3.74</td>
<td>3.93 ± 0.604</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>20.6 ± 1.58</td>
<td>27.4 ± 2.24</td>
<td>3.54 ± 0.906</td>
</tr>
</tbody>
</table>

\( a \) GRR = Ginseng Radix Rubra treatment group, as a positive control; \( b \) AM extract = \textit{A. macrosperma} aqueous extract treatment groups; \( c \) Values are mean ± S.D. of 10 mice.

Table 2. Effects of AM extract on immune functions of S180-bearing mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Concentration (mg/kg)</th>
<th>Quantitative hemolysis of sheep red blood cells (HC\textsubscript{50})</th>
<th>Lymphocyte proliferation (cpm)</th>
<th>NK cytotoxic activity (%)</th>
<th>Phagocytosis rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>–</td>
<td>424 ± 76.4**</td>
<td>11924 ± 8025**</td>
<td>41.4 ± 2.41*</td>
<td>34.5 ± 4.12***</td>
</tr>
<tr>
<td>S180 control</td>
<td>–</td>
<td>43.2 ± 9.26</td>
<td>2621 ± 1110</td>
<td>38.6 ± 2.00</td>
<td>26.0 ± 4.52</td>
</tr>
<tr>
<td>GRR a</td>
<td>2000</td>
<td>128 ± 19.5***</td>
<td>4254 ± 1261**</td>
<td>42.9 ± 3.01**</td>
<td>31.5 ± 3.83***</td>
</tr>
<tr>
<td>AM extract b</td>
<td>100</td>
<td>112 ± 11.1***</td>
<td>3985 ± 2663</td>
<td>40.8 ± 3.72</td>
<td>31.3 ± 4.33*</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>124 ± 15.6***</td>
<td>4274 ± 988**</td>
<td>44.9 ± 2.54***</td>
<td>33.8 ± 3.84***</td>
</tr>
</tbody>
</table>

\( a \) GRR = Ginseng Radix Rubra treatment group, as a positive control; \( b \) AM extract = \textit{A. macrosperma} aqueous extract treatment groups; \( c \) Values are mean ± S.D. of 10 mice; \( * \)Significantly different from S180 control group at \( p<0.05 \); \( ** \)Significantly different from S180 control group at \( p<0.01 \); \( *** \)Significantly different from S180 control group at \( p<0.001 \).

Table 1. Effects of AM extract on body and tumor weights of S180-bearing mice

The effects of AM extract on humoral immune function was estimated by measuring quantitative hemolysis of sheep red blood cells \textit{in vivo}. As shown in Table 2, a significant reduction in the assay was induced in S180 control group. AM treatments could markedly increase the antibody secreted by spleen cells in mice. The effect of 100 mg/kg dose was the best and it could restore humoral immunity in S180-bearing mice close to Ginseng Radix Rubra (GRR) 2000 mg/kg, a positive control well known as a typical immunostimulator.

b) Effects of AM extract on cellular immune function

The effects of AM extract on cellular immune function was estimated by measuring lymphocyte proliferation and NK cell cytotoxicity \textit{in vivo}. Both T cells and macrophages play a crucial role in the generation of cellular immune responses. In the assay, spleen lymphocyte proliferation and NK activity were significantly decreased in S180 control group. AM extract at 100 mg/kg and 250 mg/kg remarkably demonstrated spleen lymphocyte proliferation stimulation and increased NK activity, even higher than the functions of GRR 2000 mg/kg, whereas the dose of 50 mg/kg did not (Table 2). In NK cell cytotoxicity assay, additionally, all doses of AM administrations could significantly regulate cellular immunity close to a normal level. In two assays, the AM extract at 100 mg/kg showed the best results.

Effects of AM extract on nonspecific immune function

The effects of AM extract on nonspecific immune function was estimated by measuring the phagocytic activity of peritoneal macrophages \textit{in vivo}. Administration of AM could significantly enhance the phagocytic activity of peritoneal macrophages at all doses compared to the untreated group (Table 2). The extract at 100 mg/kg showed the most effective activity, which was close to that of the macrophages were obtained from the peritoneal exudates harvested by peritoneal lavage using sterile cold Hanks' solution. The number of CRBC ingested by macrophages was counted in an optical microscope. Besides, the percentages of macrophages that phagocytosed CRBC were determined and expressed as phagocytic rate.

<table>
<thead>
<tr>
<th>Group</th>
<th>Concentration (mg/kg)</th>
<th>Phagocytosis rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>–</td>
<td>20.3 ± 1.56</td>
</tr>
<tr>
<td>S180 control</td>
<td>–</td>
<td>20.5 ± 1.41</td>
</tr>
<tr>
<td>GRR a</td>
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</tr>
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<td>250</td>
<td>20.6 ± 1.58</td>
</tr>
</tbody>
</table>

\( a \) GRR = Ginseng Radix Rubra treatment group, as a positive control; \( b \) AM extract = \textit{A. macrosperma} aqueous extract treatment groups; \( c \) Values are mean ± S.D. of 10 mice.
normal control.

Discussion
The relation between immune states and the occurrence, growth and decline of tumor is one of the essential problems in tumor immunology. Various biological response modifiers (BRMs) such as natural products having biological activity to enhance host defense system have been considered as a useful tool to inhibit tumor growth in cancer immunotherapy. In many cases, BRMs activate immune-related cells, including NK cells, lymphokine cells and macrophages, to control cancer growth. Clinical applications are to boost the body’s general vitality or to treat a debilitating condition. Thus, the traditionally used natural resources to potentiate immune system and prevent tumor growth without direct cytotoxicity for tumor cells, may be important for cancer therapy.

Indeed, a number of studies have shown AM’s multiple actions on immune system responses. Several clinical trials on patients receiving chemotherapy or radiotherapy have found that AM significantly improves appetite, alleviate weakness, anorexia, vomiting, spontaneous or night sweat and pain, increases weight and stabilizes white blood cell counts, NK cells, IL-2 and CD4/CD8 ratio.

Others have shown that AM could improve the quality of life of the subjects by enhancing physical function and healthy transition (respondents’ amount of change in their life of the subjects by enhancing physical function and healthy transition). AM significantly improves appetite, alleviate weakness, anorexia, vomiting, spontaneous or night sweat and pain, increases weight and stabilizes white blood cell counts, NK cells, IL-2 and CD4/CD8 ratio.

Although, we did not find a significant effect of AM on the growth of ascites tumour in present study, our results for the first time showed that the aqueous extract of AM exert in vivo immunomodulatory activities on S180-bearing mice in a dose-dependent but nonlinear manner.

Generally, the assessment of immunomodulatory activity against tumor was carried out by testing the humoral, cellular and nonspecific immune responses to the antigen challenge by sheep RBCs, lymphocyte proliferation and NK cell cytotoxicity, and by macrophages function tests. The humoral defence via antibody response is mediated by B cells, while other immune cells are involved in antigen processing and immunization. The antigen–antibody complex can counteract toxin and defend the infection induced by pathogen. Cell-mediated immune defense is mediated specifically by T cells including cytoxic T cells and by the activation of natural killer cells. They can kill tumors and produce many lymphocyte factors consisting of macrophage mobile factor, lymphotxin, transfer factor and interferon, which can enhance macrophage phagocytosis and the capacity of killing target cells. In nonspecific immunity, macrophages play an important role in host defense mechanism against tumors by killing them and producing effector molecules such as nitric oxide and TNF-α, which have been recognized for their cytostasis and/or cytotoxic properties against tumor cells. In this report, AM extract showed stimulatory effect on overall immune functions in S180-bearing mice. The effect was significant in the enhancement of lymphocyte proliferations, NK cell cytotoxicity, macrophages function and the secretion of antibody, which were responsible for the inhibition of the cancer and its metastasis in many cases. These observations suggest that AM-induced antitumor effects were at least partially indirect and were associated with modulation of immune functions.

In all cases a dose of 100 mg/kg was most effective in inducing the immune functions, which were as strong as, or stronger than the positive control of GRR 2000 mg/kg, and significantly higher than the S180 control. However, immune functions did not increase significantly when the dose was increased to 250 mg/kg, indicating that there may be a threshold level of dosage. This seemed consistent with the notion that the key to the regulation of immune functions rests in the level of immune state in the body, not the level of dose. The immune level can be regulated to the normal in autoimmunity process and no effect is evident at higher doses above the limit. It was achieved by integral harmony function in which network of immune-neuroendocrine interactions was the priority. Thus, 100 mg/kg dose of AM extract seemed to be pharmacologically effective dose in S180-bearing mice as far as immunomodulatory effects were concerned. However, detailed analyses of chemical compositions are needed to determine the effective elements in the extract.

Conclusions
Based on the above results and analysis, A. macrosperma, was demonstrated to possess in vivo immunomodulatory activities in S180-bearing mice, albeit without significant activities against cancer cells, suggesting that A. macrosperma contains compounds with immunomodulatory activities. Our findings also provide empirical explanation for the traditional use of this plant in China to enhance immune systems of cancer patients. Further studies on its mode of immune action and active constituents isolation are in progress.

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