ANTITUMOR EFFECTS OF PANDANUS CONOIDEUS IN IN VITRO AND IN VIVO STUDIES

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ABSTRACT: Pandanus conoideus (Buah Merah) is exclusively grown in Papua island and its neighbour areas and indigenous people has been utilizing its extract oil (SBM) as functional food for thousand of years. We have first found relatively high quantity of carotenoids which consists of alpha- and beta-carotene as well as alpha- and beta-cryptoxanthin in SBM. Beta-cryptoxanthin is a novel micronutrient associated with reducing risk of some types of cancers. However, antitumor effect of SBM has not been well examined. We evaluated antitumor potentials of SBM in vitro and in vivo. In the in vitro study, proliferation of human non-small lung cancer cells, A549 were inhibited by SBM at more than 500 μg/mL in MTT assay. The in vivo antitumor activity of SBM was evaluated using mouse Sarcoma180, mouse Lewis lung cancer (LLC) and A549 models. In all animal model assays, SBM demonstrated significant antitumor effects in either tumor volumes or tumor weights. Experiment using nude mice in A549 assay demonstrated that antitumor effect of SBM may not be associated with immunological involvement. We conclude that Buah Merah has higher potentials in prevention of cancers, especially lung cancer.

Key words: Pandanus conoideus, Buah Merah, carotenoid, beta-cryptoxanthin, Sarcoma180, Lewis lung cancer, non-small lung cancer A549

INTRODUCTION

Pandanus conoideus called Buah Merah by native people is naturally and exclusively grown in Papua island and its neighboring areas. Buah Merah is one of varieties of Pandanaceae. The extract oil from the fruits (SBM) has been consumed as daily meal by Papuan people, probably for tens thousands years.1 It was very recent that native Papuans inhabiting at the higher mountain area in Indonesia were found taking its fruit extract as source to cooked foods. It is believed that Buah Merah, meaning red fruit, can help the Papuans to survive under cool climate and in almost naked styles. The longitudinal fruit of Buah Merah is a phalanx and grows up to 1 m in length and 10 kg in weight. The edible part is on the surface where numerous bullet-like meats with seeds in size of 5mm x 15 mm are arranged.

Analyses of SBM reveal that it is rich in lipids, carotenoids and vitamin E.2 Complete analytical method of carotenoids and xanthophils of SBM was established by a research team of Nagasaki University and showed that carotenoids of SBM comprise mainly alpha- and beta-carotene as well as alpha- and beta-cryptoxanthin.3 Beta-cryptoxanthin (3-hydroxy-beta-carotene), a pro-vitamin A is contained in wide variety of fruits and vegetables.4 Our discovery of higher content of beta-cryptoxanthin in SBM brought our attention to this novel fruit for its potential health benefits.

Recently, several epidemiological studies disclosed that beta-cryptoxanthin among major carotenoids (alpha-carotene, beta-carotene, lycopene, lutein and zeaxanthin) was the only carotenoid which was inversely associated with a reduced risk of lung cancer, particularly in current smokers.5-8 In vitro study of beta-cryptoxanthin using immortalized human bronchial epithelial cells and non-small-cell lung cancer cells indicated its inhibitory effect of cell proliferation.9
Inhibitory effect of SBM in proliferation of breast cancer cells in mice was demonstrated. In addition, there are some reports that beta-cryptoxanthin may prevent other types of cancer such as cancers of esophagus, cervix, bladder and colon and help to prevent chronic diseases such as hyperglycemia and osteoporosis.

However, potential biological effects of SBM have not been well investigated, especially for lung cancer. It is well known that lung cancer is the leading cause of cancer death worldwide. We assume that SBM may prevent and/or cure lung cancer and/or other cancers inversely associated with beta-cryptoxanthin because of its higher beta-cryptoxanthin content together with other carotenoids and nutritional ingredients.

In the present study, we examined the anti-proliferative effect of SBM in an in vitro system using human non-small lung cancer cells, A549. In addition, antitumor effects were evaluated in three mouse models using mouse Sarcoma 180, mouse Lewis lung cancer cells and A549 cells. To examine the immunological involvement in SBM for antitumor effect, nude mice were used in in vivo A549 model.

MATERIALS AND METHODS

1. Extract oil
   The extract oil of Buah Merah (SBM) was provided by PT Papua Herbal Sejahtera (Indonesia). The quantity of alpha-carotene, beta-carotene, alpha-cryptoxanthin and beta-cryptoxanthin in SBM used in the study was analyzed at School of Pharmacy, Nagasaki University in Japan and was in the range of 0.4–0.9 mg, 3.3–6.7 mg, 1.8–3.1 mg and 4.5–9.0 mg per 100 g, respectively.

2. In vitro study
   Explorative in vitro study of SBM was conducted at VisionBio (Japan). 3-(4,5-dimethylthiazol-2-yl)-diphenyltetrazolium bromide (MTT) assay was used to measure the effect of SBM on cell proliferation. Human non-small lung cancer cells, A549 were incubated in DMEM medium supplemented with 10% fetal bovine serum at 37°C in a 5% CO₂ incubator for 24 hrs before use. SBM and all-trans-retinol (Wako, Japan) as positive control were dissolved with dimethyl sulfoxide (DMSO) at a concentration of 1,000 µg/mL and diluted with phosphate buffered solution for varying concentrations of SBM and all-trans-retinol (retinol). A549 cells were seeded at 2 x 10^5 cells (90 µL)/well in a 96-well plate and treated with 10 µL of diluted SBM and retinol. After 24 hr, the cells were assayed using a MTT Cell Titer 96 Non-Radioactive Proliferation Assay kit (Promega, Japan). Cell viability is expressed as a percentage of control. All measurements were done in triplicate and values were shown in average.

3. In vivo studies
   A lethal dose of SBM was assessed by oral administration to male ICR mice with body weight of 25–27 g. No toxicity was found at dose levels of 2.0 mL and 2.5 mL per animal during two-week observation and in macroscopic examinations. Since it was considered that oral 0.5 mL dose level may not exert any adverse effect, 0.5 mL of SBM per animals was given in all animal model assays. All animal experiments including in vivo studies were conducted in accordance with Animal Care and Use Committee guidelines of Effector Cell Institute.

3.1 Sarcoma 180 assay
   Sarcoma 180 tumor cells were maintained in ICR mice by weekly intraperitoneal injection. Sarcoma cells at 1 x 10^6 were subcutaneously injected at the left axillary region of mice. Seven to 10 male ICR animals per group were allocated in each study. In the first study, 0.5mL of SBM was given orally from the following day after cell injection for consecutive 20 days. Seven days after last dosing, animals were sacrificed and tumor masses were weighed. In the second study, 0.5mL of SBM was subcutaneously injected days 1 and 11 after the Sarcoma 180 cell injection. One of the two groups was sacrificed at day 7 and another group was at day 25 for tumor weighing. Control animals were given 0.5mL of water. The inhibition percentage of tumor cell proliferation is expressed as (1-T/C) x 100, where T and C are the tumor weights of SBM treated and control groups, respectively.

3.2 Lewis lung cancer assay
   Mouse Lewis lung cancer cells (LLC) were cultured and maintained in DMEM
supplemented with 10% heat-inactivated FBS in a routine way. The total number of 1 x 10⁶ LLC cells was subcutaneously injected at the left axillary region of mice. From the following day, 0.5 mL of SBM was orally administered to female C57BL/10 mice (Nihon Crea, Japan), 10 animals per group, for consecutive 20 days. The control animals were given water. Daily mortality was recorded until all animals were dead. The size of tumor was measured in mm³ with a vernier caliper at days 7, 10, 14, 17 and 20, and tumor volumes were calculated by the formula (a x b²/2)¹, where a is the larger and b is the smaller of the two dimensions. The average survival period of control and SBM groups was calculated from survival days. The survival percentage is expressed as (T/C-1) x 100, where T and C are the average survival days of SBM and control groups, respectively.

3.3 A549 assay
Human non-small lung cancer cells, A549 were provided by the Human Science Research Resources Bank (Japan) and injected subcutaneously in mice. When the A549 tumor masses were grown, they were removed and each 3–5 mm tumor mass was implanted subcutaneously at the left axillary region of seven-week-old male BALB/c nu/nu mice (Nihon Crea, Japan), 6 animals per group. Fourteen days after implantation of tumor mass when the tumor was well proliferated, 0.5 mL of SBM. The tumor volumes were measured periodically in the same method as in LLC assay. At 61 days after the implantation, the animals were sacrificed and the tumors were removed and weighed. The inhibition percentage of tumor growth is expressed as (1-T/C) x 100, where T and C are the tumor weights of SBM and non-treated control groups, respectively.

Statistical analysis
Values are expressed as the mean ± S.D.
The significance of the differences between the controls and treated groups was calculated using Student's t-test or Welch's t-test when there were differences in F-value between the groups. Values of p<0.05 were considered to be significant.

RESULTS

In vitro study
Both SBM and all-trans-retinol (retinol) as active control showed the same trend on A549 cell proliferation. At concentrations varied from 31.25 to 125 or 250 µg/mL, proliferation of A549 cells were not inhibited compared to the control treated with the same dilution mediums. However, 500 and 1,000 µg/mL concentrations of both samples revealed drastic inhibition of cell proliferation.(Fig. 1).

![Graph](attachment:image.png)

Fig. 1 A549 cell viability in MTT assay. ATP cells were seeded at 2x103 cells/well in 96-well plates and they were treated with various concentrations of SBM or retinol. for 24 hr. Cell viability was measured using MTT kit as described under Materials and methods section.
**In vivo Studies**

In Sarcoma 180 assay (Table 1), tumor weights of SBM groups decreased as compared to the controls in both oral and subcutaneous administration studies, though in oral study there was no significant difference in the inhibition of tumor weight. Subcutaneous injection of SBM revealed significant inhibition (37.9 and 68.5%) in the tumor weights at days 7 (single dose) and 27 (twice doses) after the tumor cell injection, respectively.

<table>
<thead>
<tr>
<th>Route</th>
<th>Oral</th>
<th>Subcutaneous</th>
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<tbody>
<tr>
<td>Administration</td>
<td>Day 20</td>
<td>Day 1</td>
</tr>
<tr>
<td>Sacrifice</td>
<td>Day 27</td>
<td>Day 7</td>
</tr>
<tr>
<td>Control</td>
<td>2.53g±1.77 (10)</td>
<td>0.17g±0.08 (10)</td>
</tr>
<tr>
<td>SBM</td>
<td>1.54g±0.95 (10)</td>
<td>0.11g±0.05 (9)</td>
</tr>
<tr>
<td>Inhibition (%)</td>
<td>39.0</td>
<td>37.9</td>
</tr>
</tbody>
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Mice were subcutaneously injected with 1x10^6 cells and SBM was treated orally or subcutaneously for varying durations as described in Materials and methods section. Values are given in mean±SD. Number in parenthesis indicates number of animals. *p<0.05 (Student’s t test).

In LLC assay, the SBM-treated group showed a tendency to survive longer than the control (Fig. 2). Average survival days were calculated as 23.1±3.4 and 25.0±3.9 in the control and SBM groups, respectively. SBM oral administration caused an increase in survival period by 8.2%, though there was no significant difference compared to the control.

The tumor volumes were measured until day 20 when two animals of the control died and SBM administration was withdrawn. SBM significantly suppressed the proliferation of tumor volumes along with time after LLC injection (Fig.3).

![Survival rate of mice in LLC assay. Lewis lung cancer cells were subcutaneously injected at 1x 10^6. From the following day, 0.5 mL of SBM was orally given for 20 days.](attachment:Survival_rate.png)
Fig. 3 Inhibition on proliferation of Lewis lung cancer cells in mice. Lewis lung cancer cells were subcutaneously injected at 1x 10⁶. From the following day, SBM was orally given for 20 days and tumor volumes were measured as described in Materials and methods section. *p<0.05, ** p<0.005 (Student’s t-test)

In A549 assay, SBM oral administration revealed a remarkable trend in suppression of the proliferation of A549 cells from day 21 until the end of study. At day 55, the tumor volumes in SBM group were significantly different as compare to the control (Fig. 4).

The average tumor weights (mean±SD) at day 61 were 2.25 g ± 1.76 and 1.27 g ± 0.60 in the control and SBM groups, respectively. The inhibition percentage on tumor proliferation by SBM treatment was 43.6%, though there was no significance.

Fig. 4 Inhibition on proliferation of A549 cells in nude mice. After implantation of A549 tumor, SBM was given orally from days 14 to 60. Tumor volumes were measured as described in Materials and methods section. * p<0.05 (Welch’s t-test)

DISCUSSION

We first found out the presence of higher content of carotenoids in Buah Merah extract oil (SBM) consisting of alpha- and beta-carotenoid, and alpha- and beta-cryptoxanthin, in addition to higher contents of vitamin E and trans-fatty acid free lipids of which constitutions resemble to those of human fat.

Thus, we consider that SBM is a novel functional supplement to overcome the deficiencies in vitamins A and E and malnutrition. Furthermore, relatively high content of beta-cryptoxanthin suggests that SBM may work as chemopreventive agent against some types of cancers and/or chronic diseases associated with its deficiency. However, no comprehensive and researches for its potential effects have been conducted.

In our present studies, we focused on anti-
tumor effects of SBM. Explorative in vitro study using non-small lung cancer cell-line, SBM revealed noticeable inhibition on A549 proliferation at 500 μg/mL level in the same fashion as all-trans-retinol used as positive control. The proceeding studies were conducted in animal models using Sarcoma 180, Lewis lung cancer (LLC) and A549 cells.

In Sarcoma 180 experiments, oral administration of SBM showed a tendency of inhibition in tumor weight which was measured 7 days after cessation of 20-day administration. On the contrary, either one or two times subcutaneous injection of SBM demonstrated a significant inhibition on Sarcoma 180 cell proliferation. These results suggest that continuous and/or parenteral administration may enhance antitumor effect of SBM. In LLC assay, prolonged survival time was observed and tumor volumes were significantly suppressed by SBM treatment while SBM was given for 20 days. However, the average survival days in the entire experiment period of 31 days did not increase. This may be due to withdrawal of administration of SBM. Nude mice were used in A549 cell model to examine the involvement of T cells. The proliferation of A549 cells was remarkably or significantly inhibited with regard to tumor volumes. The decrease in tumor weights was evident at 43.6% inhibition by SBM administration. Thus, SBM demonstrated noticeable suppression on A549 cell proliferation in nude mice. It suggests that T cells may not be associated with inhibitory mode of actions of SBM.

Dr. Lian et al. (2006) reported that approximate 0.5 μg/mL of beta-cryptoxanthin inhibited viability of A549 cells in in vitro assay. The concentration of SBM 500 μg/mL was equivalent to approximately 0.02 μg/mL of beta-cryptoxanthin that caused a decreased viability in the present A549 MTT assay. This indicates that not only beta-cryptoxanthin but also other ingredients in SBM may be associated with inhibition on tumor cell proliferation, and that they may work in a synergic way, though many investigations remains elucidated. However, a cohort study in China elucidated that more than 3.01 μg/dL of beta-cryptoxanthin in blood level were significantly associated with a reduced risk of lung cancer, especially in smokers.

Safety of SBM has been confirmed by in vitro mutagenicity tests, and acute and subacute toxicity studies using rats (Nishigaki T et al. submitted for publication). In addition, SBM has a long history of use by humans in Papua island, Indonesia. From these facts, we conclude that SBM has antitumor effects and is safe for human consumption. We also expect that Buah Merah and its extract oil will provide health benefits as a novel food supplement. In order to well-understand the features of SBM as a potent chemopreventive supplement against lung and/or other cancers, we carry out pharmacokinetic study and examine pharmacological mechanisms in actions of SBM in animal models as well as human subjects. In our current studies, we have been investigating the antitumor mode of action of SBM immunohistochemically using the tumors obtained from present studies.

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(A part of result from the studies was reported at International Conference of International Society for Nutraceuticals and Functional Foods (ISNFF) 2010, Bali, Indonesia.)