Anticancer activity and immunostimulating effect on NK-cell activity of a well-known Thai folkloric remedy

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There are strong evidences supporting folklore about medicine, in particular, those having a historical reputation as anticancer agents that represent promising candidates for chemotherapeutic research and development. The objective of the present study is to investigate the anticancer and immunostimulating activities of one Thai folkloric remedy for the treatment of cancer *in vitro* and *in vivo*. The cytotoxic activity of the remedy was investigated *in vitro* in KB cells and *in vivo* in mammary cancer-bearing rat (induced by 7, 12 DMBA at a single oral dose of 150 mg/kg body weight) and in cervical cancer xenograft nude mouse models. Acute and subacute toxicity tests were performed following intraperitoneal and/or oral dose administration. In the rat model, 30 rats received a daily dose of distilled water (control group) or the remedy at a daily dose of 1,500 mg/kg body weight (treated group) by intragastric gavage. In the mice model, 16 mice received a daily dose of distilled water (control group) or intraperitoneal injection of two doses of 1 mg/kg body weight mitomycin C (positive control) or the remedy at a daily dose of 2,580 mg/kg body weight (treated group) by intragastric gavage for 60 days. Splenic mononuclear cells from nude mice treated with the remedy at a dose of 2,580 mg/kg body weight/day for 1, 2, and 3 weeks were investigated for their NK cell activities by a 4 h ^{51}Cr release assay, in comparison with that of the corresponding control groups. Based on the criteria for cytotoxic activity proposed by the National Cancer Institute of USA (IC_{50} < 30 μg/ml), the cytotoxic activity of the remedy (IC_{50} > 100 μg/ml) was considered weak (IC_{50} > 100 μg/ml). The remedy was well tolerated in both acute and subacute toxicity tests. The remedy was shown to exert significant anticancer activity in both animal models. In the rat model, the 180-day survival rates of rats in the treated group (53.57%) were significantly higher than the untreated group (53.57 *vs.* 6.89%, p < 0.01) and the survival time was significantly longer than that of the untreated group (173 (133-220) *vs.* 119 (90-143) days, p < 0.01]. In addition, the lung metastasis rate was significantly lower in the treated group (10.17 *vs*. 20.69%). In mice model, significant inhibition of tumor volume was observed in mice treated with the extract from day 10 to day 60 (T/C =50-75%, with maximum activity on day 28) compared with the control (p < 0.05). The NK cell cytotoxic activity (%) in mice treated with the remedy for 1 or 2 weeks was significantly increased (by about 8.26-57.97% and 13.3-42.7%, respectively) compared with the control. The remedy may exert its anticancer activity by acting as an immunostimulating agent through activating NK cell activity.

Keywords: Cancer, chemotherapy, traditional medicine, mammary cancer immunostimulatign effect.

INTRODUCTION

Cancer is one of the major causes of death and it constitutes the greatest health care problem since ancient times. Cancer chemotherapy currently plays a significant role in the treatment of cancer which may be either curative (by itself or used as an adjuvant to surgery and/or radiation) or palliative, depending on the specific types of tumors. Even with the progress in modern medicine, people in some parts of the world still culturally and religiously adhere to the traditional approach of health care. By experience, mankind has learned ways of dealing with pain and illness. In Thailand, the use of folk medicine is common not only in the rural areas where modern medicine is almost unobtainable but also in urban areas where a large number of the population still prefer to see herbalists or traditional healers rather than physicians for their ailments, especially cancer. Very commonly, cancer patients are treated with a mixture of herbs or herbal decoction, concurrently with some form of ritualistic ceremony and incantation. These practices are often coupled with misinformation and in some instances, lack of knowledge about modern medicine. Most of the folk medicines have not been supported by experimental investigation in vitro and in animals. Patients are therefore subject to possible risks of toxicity. In order to substantiate their claims of cure, it is essential that the treatment of herbalists, traditional healers, or even traditional practitioners be scientifically proven.

There are strong evidences supporting folklore about medicine, in particular those having a historical reputation as anticancer agents that represent promising candidates for chemotherapeutic research and development. Examples of plant-derived compounds that have been registered for use as anticancer drugs include vincristine, vincristine, etoposide, teniposide, paclitaxel, navelbine, taxotere, topotecan, and irinotecan (Dholwani et al., 2008). In the present study, one of the most popular Thai folkloric remedies for treatment of cancer prescribed by medicinal practitioner Dr. Sommai Thongprasert (private cancer clinic, Singburi Province) has been selected for investigation of its anticancer including immunostimulating activities in vitro and in vivo. This folkloric remedy has gained increasing impression and acceptance among local Thai cancer patients as well as those from neighboring countries such as Malaysia and Singapore. A full set of the remedy consists of parts from five plants, and five animals.

MATERIALS AND METHODS

Chemicals and reagents

Eagle's Minimum Essential Medium (EMEM), RPMI 1640 medium, fetal bovine serum albumin, Triton-X 100, and Hank's Balanced Salt Solution (HBSS) were purchased from Gibco BRL Life Technologies (Grand Island, NY, USA). Penicillin, streptomycin, ampicillin trihydrate, and mitomycin C (MCC) were purchased from Dumex Co. Ltd. (Bangkok, Thailand). Folin-Ciocalteau phenol reagent, 5-fluorouracil (5-FU), and 7, 12-dimethylbenz (a)anthracene (7, 12-DMBA) (Sigma Chemical Co. Ltd., USA) was purchased from Sigma Chemical Co. Ltd. (USA).

Preparation of plant extracts

One set of the remedy (an average dry weight of 1,200 g) consists of a mixture of parts from five plants, and five animals as follows: (a) Plants: *Polygala chinensis* (Ya Peek Kai Dam, Polygalaceae family, 60 g, whole part), *Ammania baccifera* (Fai Duen Haa, Lythraceae family, 60 g, whole part), *Clinacanthus nutans* (Phaya Yor or Phaya Pling Thong, Acanthaceae family, 35 g, stems and leaves), *Canna indica* (Puttaraksa, Cannaceae family, 600 g, rhizomes), *Smilax corbularia* (Khao-Yen-Nuea, Smilacaceae family, 70 g, rhizomes), and (b) Animals: *Manis javanica* (Nim, 50 g, scales), *Hystrix brachyuran* (Menn, 5 g, spines), *Damonia subtrijuga* (Tao Naa, 95 g, carapaces), Dasyatis spp. (15 g, spines), *Trionyx cartilajineus* (Ta Pabb Nam, 35 g, sternums).

Information on plant species, parts used, including dose regimen used for the treatment of mammary cancer was kindly provided by Dr. Sommai Thongprasert. Plant materials were grown in various parts of Thailand and collected during particular seasons. Authentication of plant materials were carried out at the herbarium of the Department of Forestry, Bangkok, Thailand where the herbarium vouchers have been kept. All materials were washed thoroughly with water and cut into small pieces before being mixed in a pot (60 cm in diameter). Preparation of the water extract of the remedy was performed by boiling all materials in 10 litres of water at 100^{o}C until the extract (decoction) evaporated to a volume of 0.5 litre. The collected decoction was filtered through a filter paper (Whatman number 1) under vacuum and centrifuged at 7,000 x g for 30 min (HTS International Equipment Co. Ltd., MS, USA). The process was repeated daily for 15 consecutive days. The decoction was concentrated by evaporation under reduced pressure at 55-65^{o}C in a vacuum evaporator (Eyela, Tokyo Raikkaai Co. Ltd., Tokyo, Japan). All of the 15-day concentrates were pooled and lyophilization was applied for complete dryness (Vertris, Research Equip-

ment Gardiner Co. Ltd., NY, USA). The dark brown lyophilized remedy was ground into powder and stored at 4ºC until use. The average dry weight of lyophilized powder obtained from each set of the remedy following a 15-day water extraction was 193 gram. Assuming the average patient's body weight of 60 kg, the therapeutic prescribed dose in humans is approximately 215 mg/kg body weight/day.

In vitro assay for cytotoxic activity

Cell line and culture

KB, a cell line derived from human carcinoma of the nasopharynx (provided by Virus Research Institute, Department of Medical Science of Thailand) was used for the assessment of cytotoxic activity of the remedy (Oyama and Eagle, 1956). The cell line was maintained in a 75-ml plastic tissue culture flask (Nunc, Intermed Co. Ltd., Copenhegen, Denmark) in EMEM medium supplemented with 10% heated fetal bovine serum and 100 IU/ml of penicillin and 100 μg/ml of streptomycin sulfate and were maintained in a 5% CO_2 atmosphere (37°C) with 95% humidity.

Cytotoxic assay

The cells were seeded in a 96-well plate at a density of 4 x 10^4 cells/well in 100 μl culture medium. Following a 24 hrs incubation, cells were treated with varying concentrations (0, 25, 30, 50, and 100 μg/ml) of the remedy and 5-FU (positive control, 10 μg/ml) for 96 h. Following washing, cell viability was determined by tryphan blue exclusion staining and cells at a density of 4 x 10^4 cells/ml were prepared. Cell proliferation (%) was measured through the determination of protein concentration according to the method of Oyama and Eagle (Huggins et al., 1961). The IC_{50} (concentration of the remedy that inhibited cell proliferation by 50%) value was calculated using the CalcySyn™ (Biosoft, USA) software. The results were generated from three independent experiments and each experiment was performed in triplicate.

Animals

Outbred Wistar albino rats of both sexes (6 weeks of age, weighting 120-130 g) and Balb/c male nude mice (6-8 weeks of age, weighting 17-22 g) were purchased from The National Laboratory Animal Centre of Thailand. They were housed under standard conditions and fed with a stock diet and water *ad libitum.* Approval of the study protocol was obtained from the Ethics Committee for Research in Animals, Mahidol University, Bangkok, Thailand.

Anticancer activity against mammary cancer in rats

Acute and subacute toxicity tests

Acute and subacute toxicity tests of the remedy were performed following intraperitoneal and oral dose administration.

Acute toxicity test

For intraperitoneal dose administration, the remedy at doses of 400, 500, 1000, 1500, 2000, 2500, 3000, and 4000 mg/kg body weight (in 1 ml normal saline) were injected to the rats in each dosing group (10 males and 10 females for each group). The vehicle control group received 1 ml of normal saline. For oral dose administration, the remedy at doses of 4000, 8000, 10000, and 15000 mg/kg body weight (in 2 ml distilled water) were given to the rats in each dosing group (10 males and 10 females for each group) by intragastric gavage. The vehicle control group received 2 ml of distilled water. Animals were closely observed for signs of toxicity during the first 30 min, periodically during the first 24 hours, and then daily for 14 days. The LD_{50} (lethal dose 50%) value was calculated using the CalcySyn™ (Biosoft, Kent, USA) software.

Subacute toxicity test

The remedy at a dose of 1,500 mg/kg body weight (in 2 mldistilled water) was given to 20 rats (10 males and 10 females for each group) by intragastric gavage daily for 6 months. The vehicle control group (10 males and 10 females) received 2 ml of distilled water. Animals were closely observed for signs of toxicity during the first 30 min, periodically during the first 24 hours, and then daily for 6 months. Food and water consumption were recorded daily and blood samples were collected from the caudal vein at 0, 1, 4, 8, 12, 16 weeks, and 6 months interval for hematology (CBC and hematocrit) and biochemistry (total protein, albumin, globulin, creatinine, SGOT, SGPT, and alkaline phosphatase) tests. At the end of the observational period, all animals were sacrificed under ether anesthesia and vital organs (brain, heart, kidneys, liver, spleen, stomach, large and small intestine, and lungs) were removed from all animals for gross and histopathological examination.

Induction of mammary cancer in rats

Mammary cancer was induced in 230 female Wistar albi-

no rats (aged 50-55 days, weighing 160-170 g) *via* intragastric fed with a single oral dose of the remedy at a dose of 150 mg/kg body weight (in 2 ml corn oil) (Huggins et al., 1961). In the vehicle control group, 30 rats of the same age and body weight ranges were fed with 2 ml of corn oil. Each rat was weighted and mammary glands palpated twice a week for 250 days for the appearance of subcutaneous masses of mammary tumors. Tumor size was obtained by averaging the largest and the perpendicular diameters using calipers with accuracy of 0.1 mm.

Assessment of anticancer activity in 7, 12-DMBA-induced mammary cancer in rats

Rats which developed at least one mammary tumor within 180 days after induction were randomized to two groups (30 rats each) to receive a daily dose of distilled water (2 ml, control group) and the remedy at daily doses of 1,500 mg/kg body weight (2 ml in distilled water, treated group) by intragastric gavage. This dose is equivalent to the therapeutic dose used in humans given at the same route and schedule (7 times of the human dose for rat) (Owens, 1963). The dose was adjusted weekly for change in the body weight. Efficacy parameters included tumor size inhibition, survival time, and survival rate of the tumor-bearing rats within the six months period. The malignancy stage of the tumors was confirmed at autopsy by histopathological examination.

Anticancer activity against cervical cancer in xenografted nude mice

Toxicity test

Acute and subacute toxicity of the remedy were investigated in 24 (12 each) male Balb/c nude mice at a single dose of 2,580 mg/kg body weight (in 0.2 ml distilled water) and daily doses of 2,580 mg/kg body weight for 90 days by intragastric gavage (human therapeutic equivalent dose for mice), respectively. Control mice consisted of 6 mice fed with distilled water (0.2 ml). Animals were weighted daily.

Human cervical cancer xenografted nude mice

The human cervical tumor was transplanted into donor nude mice (Picha et al., 1988) until the required size developed (8-12 weeks). The donor mice were killed by $C0_2$ aspiration and the tumors were aseptically removed and transferred to the watch glass containing cold MEM media. The explant was minced into small pieces of solid tumor blocks of about 1 mm^3 in volume and xenografted into the right flank of the 115 recipient nude mice. Tumor size was measured in three dimensions by caliper and tumor volume was calculated from the formula: $\pi/6 \times a^2b$, where **a** is the smallest diameter and **b** is the largest diameter (Stabb and Anderer, 1982). The histopathological appearance of the tumor was confirmed after autopsy. To prevent infection, amplicillin trihydrate in sterile distilled water (500 mg/l) was added in drinking water for one week. Fourth serially passaged xenografts from the original cervical cancer were used to evaluate the anticancer activity of the remedy. After 3 weeks of serial transplantation, 89 mice developed tumors with tumor volume varying from 1 to 30 mm^3.

Assessment of anticancer activity in cervical cancer xenografted nude mice

Of the 89 mice which developed tumors (tumor size 1-30 mm^3), 48 transplanted nude mice were selected for the study of anticancer activity of the remedy based on the following criteria: healthy, with gradual tumor growth during the 3 weeks period, and with tumor volume 7-16 mm^3. Mice were allocated to three groups (16 mice each) as follows: treated (oral dose of the remedy at 2,580 mg/kg body weight/day for 60 days), untreated (fed with distilled water daily for 60 days), and positive control [intraperitoneal injection of two doses of mitomycin-C (MMC: 1 mg/kg body weight)] on day 1 and day 5 every other week for 3 courses. This dose is equivalent to the therapeutic dose in humans given at the same route and schedule (12 times of the human dose for mice) (Owens, 1963). The dose was adjusted daily depending on body weight change. Tumor size was measured twice a week and tumor volumes were calculated. Results are expressed as T/C% against time, where T is the mean volume of the test group and C is the mean tumor volume of the control group. Criteria for anticancer activity is T/C% < 75% (Geran et al., 1972).

Autopsy and histopathological examination

For toxicity and anticancer activity evaluation in the rat and the mouse models, all internal organs were removed from the animals at autopsy and observed macroscopically. Samples were fixed with 10% formalin solution. Specimens were washed in phosphate buffer three times, then dehydrated in an ascending series of ethanol for 15 min each and embedded in paraffin, followed by sectioning and staining with hematoxylin and eosin.

Stimulatory effect on natural killer cells

The stimulatory effect of the remedy on natural killer (NK) cell activity was evaluated in 54 nude mice. The animals

were allocated to six groups to receive treatment with the remedy at a dose of 2,580 mg/kg (in 0.2 ml distilled water, treated group) and distilled water (0.2 ml, untreated control group) for 1, 2, and 3 weeks (10 mice per group).

Preparation of splenic mononuclear cell suspension

On the day of the experiment, spleens were removed aseptically from all the mice and transferred to a sterile petri-dish with cold RPMI 1640 culture medium. The spleens were cut into small pieces and the bottom of the sterile test tube was used to tease the splenic pieces through a sterile stainless steel sieve into a 1-3 ml RPMI 1640 culture medium. The splenic mononuclear cells used as effector cells (NK cells) were separated using a Ficol-Hypaque mixture (Boyum, 1968). Cells were adjusted to the desired density with a RPMI 1640 medium supplemented with 3% fetal bovine serum for cytotoxic assay.

Natural killer cell cytotoxic assay

NK cell cytotoxic assay was performed using Yac-1 lymphoma cell line, a Moloney virus-induced lymphoma in A/Sn mice (Cikes et al., 1973). The splenic mononuclear cells used as effector cells (NK cells) were checked for viability (> 95%) using tryphan blue dye exclusion. Cells were adjusted to a density of 1×10^7 cells/ml with a RPMI 1640 medium supplemented with 3% fetal bovine serum. The ^{51}chromium (^{51}Cr) release cytotoxic assay was performed in a 96-well flat bottom micro-titer plate according to the previously described method (Cikes et al., 1973) with some modifications. The assay performed the effector: target (E:T) ratios of 100:1, 50:1, and 25:1. In each experiment, quadruplicates of maximum release and spontaneous release wells were determined simultaneously. The maximum release well contained 1×10^4 labelled target cells (100 μl) and 100 μl of 20% Triton X-100. The spontaneous release well contained 1×10^4 labelled target cells (100 μl) and 100 μl of 3% RPMI 1640 medium supplemented with 3% fetal bovine serum. After thorough mixing, the plate was gently shaken and centrifuged at 200xg (25°C) for 5 min and then incubated for 4 h (37°C) in a 5% CO_2 atmosphere with 95% humidity. To collect the radioisotope released into the supernatant from each well, the plate was centrifuged at 200xg at 4°C for 5 min and put in an appropriate labeled vial for ^{51}Cr radioactivity count in gamma counter (Gamma 5500 Beckman Irvine, CA, USA). Cytotoxic activity (%) was determined using the formula:

$$\% \text{ Cytotoxic activity} = \frac{\text{Experimental release - Spontaneous release}}{\text{Maximum release - Spontaneous release}} \times 100$$

Experimental release is the mean count *per* min (*cpm*) of supernatant from the quadruplicate wells containing a mixture of effector and labeled target cells. Maximum release is the mean *cpm* of supernatant from the labeled target cells incubated with 20% Triton X-100. Spontaneous release is the mean *cpm* of supernatant from the labeled target cells incubated in complete RPMI 1640 medium.

Statistical analysis

All quantitative variables were presented as median (range). Comparison of the qualitative and quantitative variables between the two independent groups was performed using chi-square and Mann-Whitney U test, respectively. Statistical significance level was set at $\alpha = 0.05$ for all tests.

RESULTS

***In vitro* cytotoxic activity**

The remedy at concentrations of 25, 30, 50, and 100μg/ml inhibited growth of KB cells to 73.3, 71.2, 68.9, and 50.9%, respectively. The IC_{50} of the remedy in KB cells was greater than 100μg/ml.

Toxicity and anticancer activity against mammary cancer in rats

Acute and subacute toxicity

The LD_{50} of the remedy following intraperitoneal dose administration was 1,350 mg/kg body weight. No rats died following an oral dose of up to 15,000 mg/kg body weight. Sedation and unusual locomotion were observed in some rats on the first day of intraperitoneal dosing but recovered within 1-2 days. Oral administration of the remedy at a dose of 1,500 mg/kg body weight for up to 6 months was well tolerated with no significant changes in body weight, food and water consumption, blood haematology and biochemistry, and gross and histopathology. All rats survived up to a period of 6 months.

Anticancer activity in 7, 12 DMBA-induced mammary cancers in rats

Development of mammary cancer

Mammary cancer developed in 118 out of 230 rats (51.44%) following an oral dose of 150 mg/kg body weight 7, 12-DMBA. The earliest tumor development

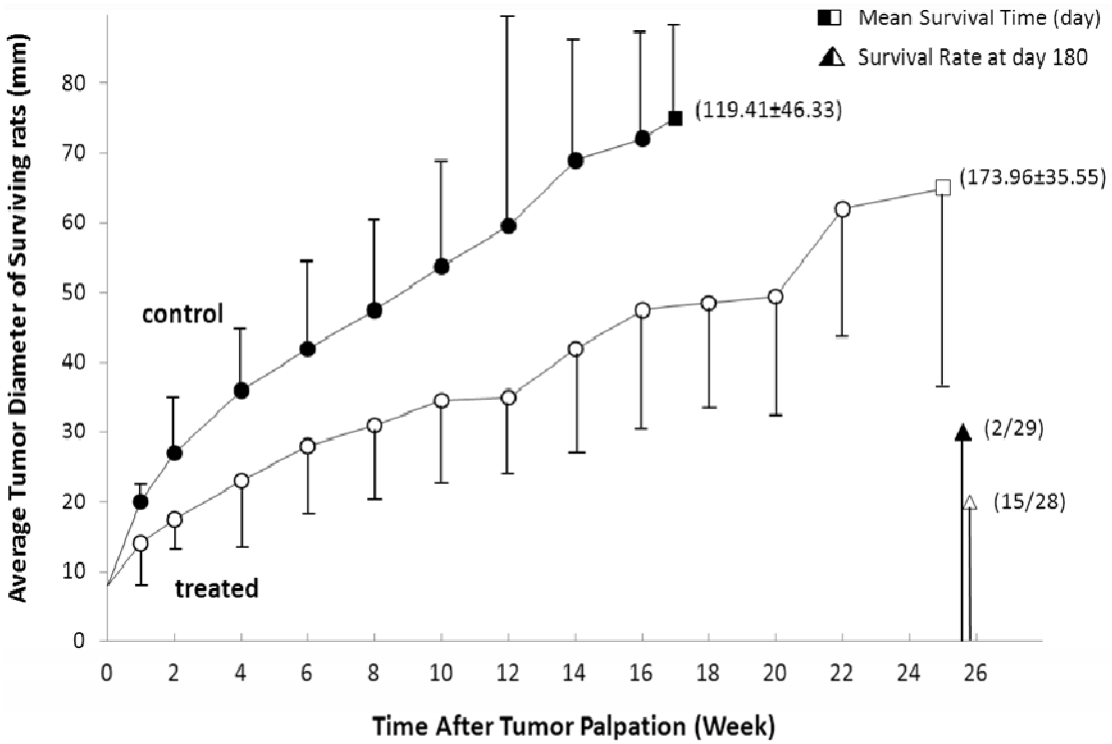

Figure 1. Anticancer activity as indicated by the prolongation of survival time, increase of survival rate, and inhibition of tumor growth) of the remedy in 7, 12 DMBA-induced mammary cancer model in rats. Data are presented as median and range values.

occurred as early as 46 days after induction. Twenty-two (9.57%) rats died within two weeks. The causes of death in most cases were anemia, leukopenia, and infection (lung abscess). No tumor or death was observed in rats fed with corn oil. The carcinogen, 7, 12-DMBA induced five types of mammary tumors, *i.e.,* adrenocarcinoma (72.05%), fibroadenoma (5.5%), adenosis (3.73%), fibroma (1.24%), and intraductal papilloma (0.63%). The predominant type, adenocarcinoma, appeared in all differentiation grades, *i.e*., tubular formations, comedo forms, and papillary and cribriform carcinoma. Twenty (16.94%) rats developed more than one type of tumor or tumor with lung metastases.

Anticancer activity evaluation

Anticancer activity against mammary cancer of the remedy was evaluated in 28 (treated group) and 29 (untreated control group) rats with confirmed malignant adenocarcinoma of mammary glands by histopathological examination at autopsy/necropsy. Significant inhibition of tumor growth and reduction of tumor size (compared with control group) were observed during the first four weeks of treatment with the remedy (Figure 1). The 180-day survival rates of rats in the treated group (15/28, 53.57%) were significantly higher than (p < 0.01) that of the untreated group (2/29, 6.89%). In addition, the median (range) survival time was significantly longer (p < 0.01) in the treated group [173 (133-220) days] than that of the untreated group [119 (90-143) days]. Lung metastasis rate was significantly lower in the treated group compared with the control group [3 (10.17%) *vs*. 6 (20.69%)].

Toxicity and anticancer activity in nude mice

Toxicity

In the acute toxicity test, no mice died within the 14 days observation period. Results of subacute toxicity test in nude mice showed that the body weights of mice treated with the remedy at a dose of 2,580 mg/kg body weight/day were significantly higher (median = 21.5 g) than the untreated mice (median = 26.5 g) at 90 days of observation period (p < 0.05). One mouse each in the treated and untreated group died on day 82 and day 77, respectively. Histopathological examination revealed pneumonia and atelectasis in the lungs, dilated blood vessels and congestion in the kidneys, and lymphoid follicle hyperplasia in the spleen of both treated and untreated groups in both acute and subacute toxicity tests. However, spleen infarction and spleen atrophy

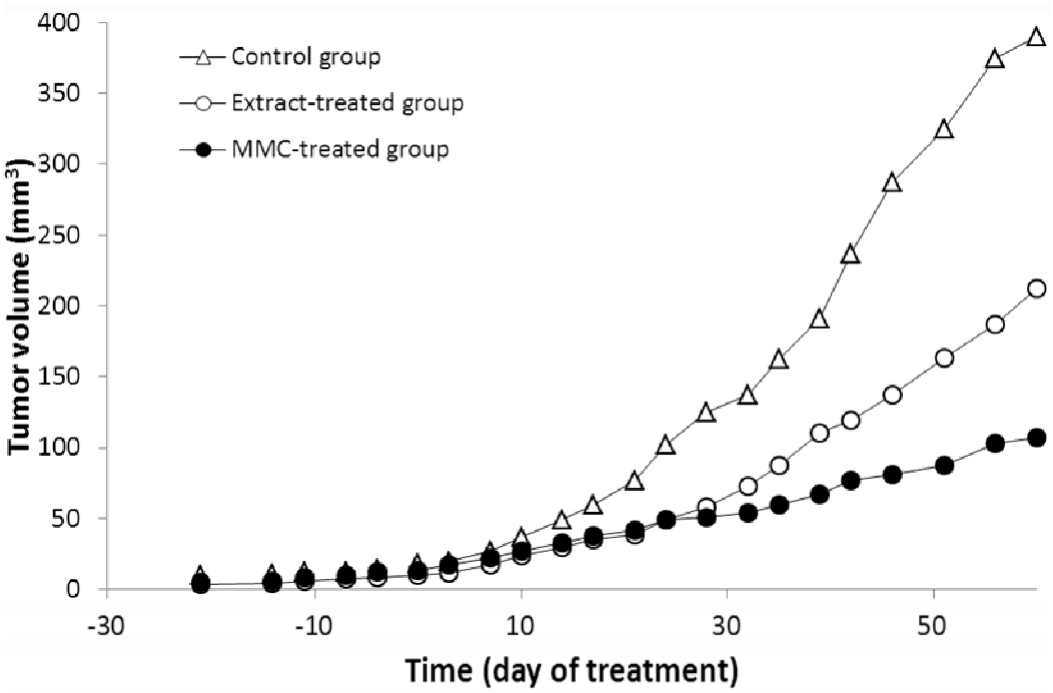

Figure 2. Median tumor volume of xenografted mice treated with the remedy (extract) and MMC, in comparison with the control (fed with distilled water).

were found in one mouse each in the remedy treated group.

Anticancer activity in xenograft mouse model

Histopathological examination showed that the tumors grown in all nude mice were squamous cell carcinoma of the mammary glands as the original human cells.

Tumor growth

Figure 2 shows the median tumor volumes in the three groups of mice treated with the remedy, MMC, and untreated control. The initial tumor volumes in all groups were comparable. Significant inhibition of tumor volume was observed in mice treated with the extract from day 10 to day 60 (T/C =50-75%, with maximum activity on day 28) and mice treated with MMC from day 14 to day 60 (T/C =27-75%, with maximum activity on day 60), compared with the control ($p < 0.05$). There was no significant difference in the tumor volumes of mice treated with the remedy and MMC during the 60 days observation period. Based on the NCI criteria for antitumor activity (%T/C < 75), both the remedy and MMC were considered to possess active anticancer activities from day 7 and day 14 onwards, respectively (Figure 3). There was no significant difference in tumor growth rates of the control mice.

Survival rate

All mice treated with the remedy survived until the end of the observation period (60 days). On the other hand, 5 out of the 16 mice treated with MMC died.

Growth rate

The body weight of mice was used as a parameter to assess the growth rate of the xenografted mice in the three groups (Figure 4). The body weight of mice treated with the remedy was significantly higher than that of the MMC-treated mice and the control group during day 28 to day 60 ($p < 0.05$). The body weight of the MMC-treated mice was significantly lower than the control during day 32 and day 60 ($p < 0.05$).

Stimulatory effect on nude mice natural killer (NK) cells *in vitro*

Splenic mononuclear cells from nude mice treated with the remedy at a dose of 2,580 mg/kg body weight/day for 1, 2, and 3 weeks were investigated for their NK cell activities by a 4 h ^{51}Cr release assay, in comparison with that of the corresponding control groups. The cytotoxic activity (%) in mice treated with the remedy for 1 or 2 weeks were significantly increased (by about 8.26-57.97% and 13.3-42.7%, respectively) compared with the

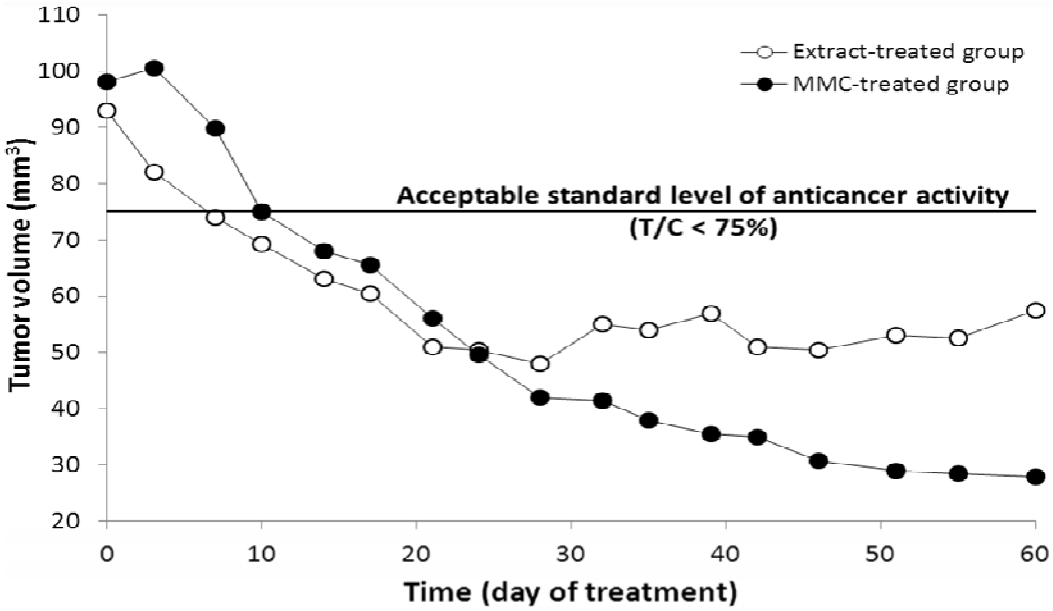

Figure 3. Percent reduction of T/C across time in the three groups of xenografted mice treated with the remedy (extract) and MMC.

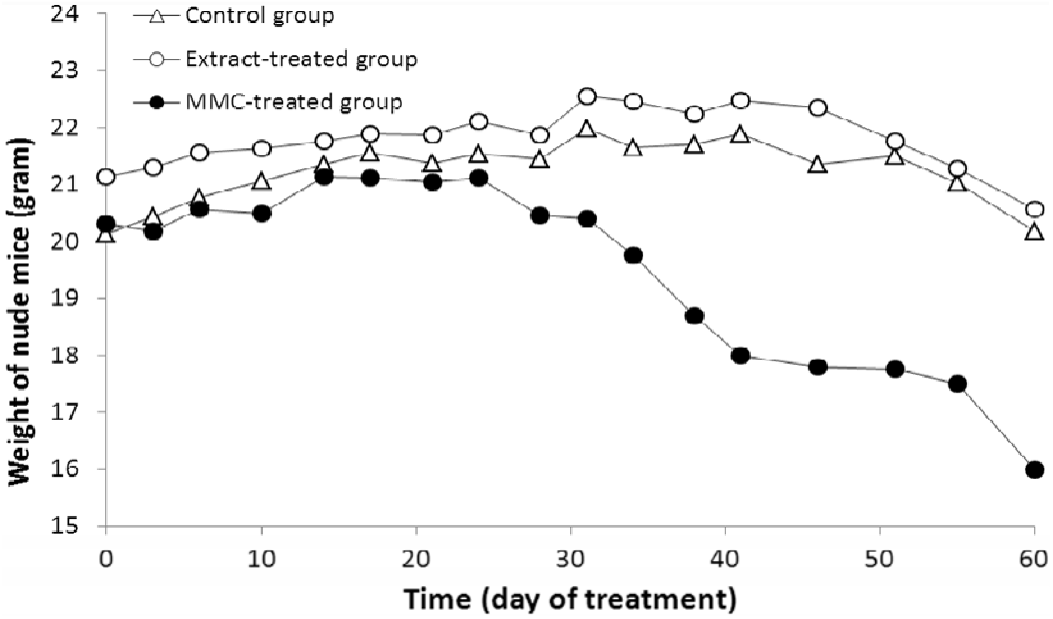

Figure 4. Median body weight of xenografted mice treated with the remedy (extract) and MMC, in comparison with the control (fed with distilled water).

control. There was no significant difference in cytotoxic activity in mice treated with the extract for 3 weeks (Figure 5).

DISCUSSION

The cytotoxic activity of the remedy was investigated *in vitro* in KB cells and *in vivo* in mammary cancer-bearing rat (induced by 7, 12 DMBA) and cervical cancer xenograft nude mouse models. Based on the criteria for cytotoxic activity proposed by the National Cancer Institute of USA ($IC_{50} \leq 30$ μg/ml) (NCI, 1959), the cytotoxic activity of the remedy was considered weak ($IC_{50} > 100$ μg/ml). On the other hand, the remedy showed promising anticancer activity in both *in vivo* mod-

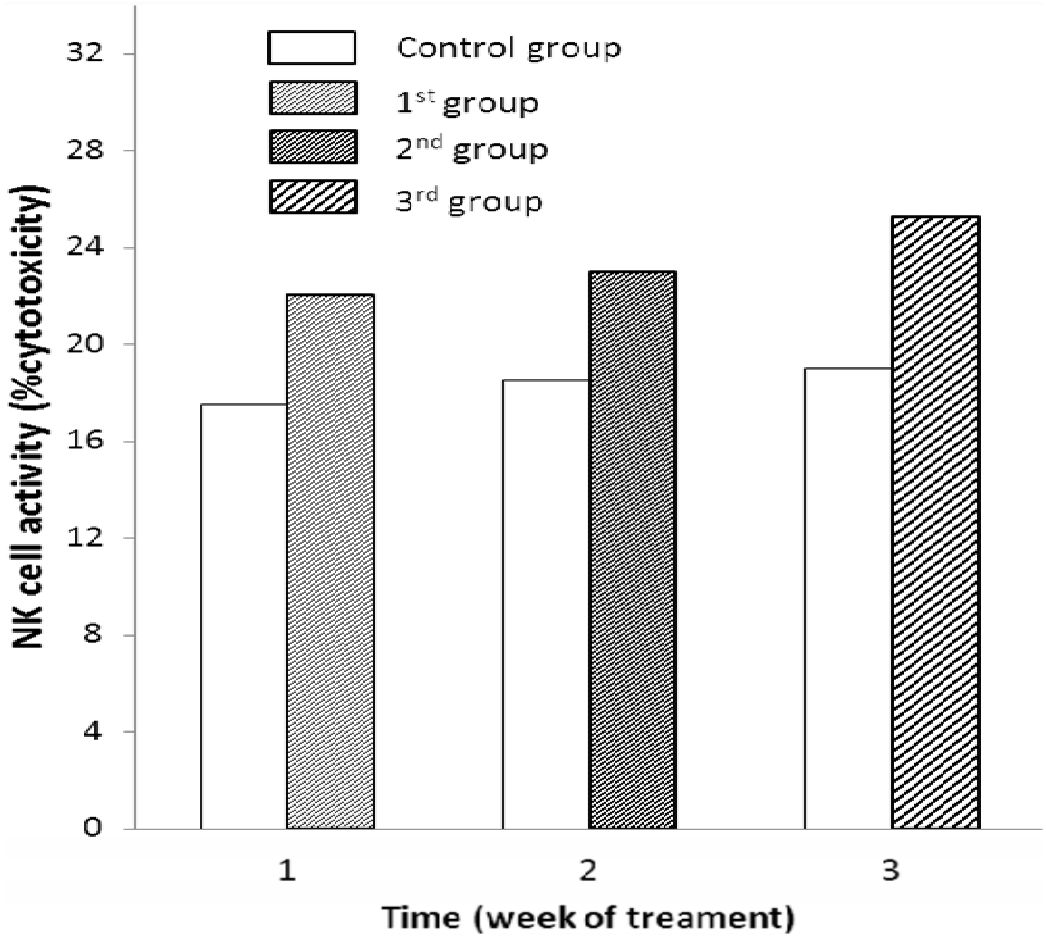

Figure 5. Cytotoxic activity of NK cells in the three groups of mice fed with the remedy for 1, 2, and 3 weeks, compared with the corresponding control mice. Data are presented as median values.

els. In the rat mammary cancer model, daily feeding of the remedy at the therapeutic equivalent prescribed dose of 1,500 mg/kg body weight significantly prolonged survival time (173 *vs*. 119 days), increased survival rate (53.57 *vs*. 6.89%), and delayed tumor growth of the rats compared with the control. In addition, the remedy reduced the metastatic rate (to lungs) by about 50%. Tumor growth inhibition was clearly seen in the early four week period when the tumors were only small in size. When they become larger, they might have developed cirrhous materials which acted as barrier to the remedy. 7, 12 DMBA-induced mammary cancer in rats was used as a model for assessing the anticancer activity of the remedy as a potent carcinogen, inducing high proportion of malignant adenocarcinoma in rats with relatively low initial lethality. Previous investigations have demonstrated that rat mammary adenocarcinoma rarely metastasizes. In one study, only 1.96% of lung metastasis was observed following a dose of 130 mg/kg body weight 7, 12-DMBA (Cancer Chemotherapy National Service Center (1959). The relatively high lung metastasis rate observed in our study of 8.93% may be due to the use of a relatively high dose (150 mg/kg body weight).

The fourth serially passaged human cervical cancer xenografts from the original cervical cancer were used as an additional model to assess the anticancer activity of the remedy. This cancer model has been used as a standard model for assessing anticancer activity of the candidate compounds and plant extracts (Guiliani et al., 1971; Inbra et al., 1989). Administration of the remedy significantly reduced tumor growth with the T/C ratio of < 75% starting from day 7 to day 28 after treatment. Thereafter the tumor growth was relatively stable until the end of the treatment period (day 60). In contrast, a gradual decrease of T/C ratio was observed in the MMC-treated group from day 14 until day 60 (Figure 3). In addition, the remedy significantly increased the growth of mice (Figure 2). All mice survived until day 60 of the treatment. This observation was in agreement with that observed in 7, 12 DMBA-induced mammary cancer model in rats, where the remedy was also found to significantly decrease tumor growth in the first four weeks of treatment. Noteworthy, the remedy exerted its anti-

cancer activity (both mammary and cervical cancer) in the absence of cytotoxic activity. Thus, the therapeutic effect of the remedy was not cytolytic in nature. In contrast, only one-third of the 16 mice treated with MMC survived, suggesting the possibility of cytotoxic activity of MMC. Serious adverse effects of MMC commonly found in the treatment of cancer patients include myelosuppression characterized by marked leukopenia and thrombocytopenia including nausea, vomiting, diarrhea, and stomatitis (Andrez, 2009). The remedy was well-tolerated both in rats and mice following the acute and subacute toxicity tests. In rats, no mortality was observed up to a single dose of 15,000 mg/kg body weight, indicating relatively harmless toxicity (Loomis, 1968). Subacute toxicity test revealed no unusual functional disorder of important organs (hemopoietic, liver, and kidney). In a preliminary study (Pornsiriprasert, 1986) where only the plant parts (5 plants) were used in the remedy, oral administration of the remedy at a dose of 1,000 mg/kg body weight daily for 6 months to mammary cancer-bearing rats significantly increased survival rate (43.75 *vs*. 20%) and prolonged survival time (176.22 *vs.* 150.87 days) without significant reduction of tumor weight (57.33 *vs*. 41.18 g). This suggests that the addition of the animal parts enhanced the anticancer activity of the remedy against mammary cancer. The rhizomes of *Similax corbularia*, a climbing vine distributed in Southeast Asia, has been prescribed by Thai traditional practitioners for the treatment of breast and ovary cancers, AIDS, septicemia, and lymphatic diseases (Tewtrakul et al., 2006). The leaves of *Ammannnia baccifera*, a plant commonly distributed throughout Asia, often as a weed in rice fields, is known to possess a broad spectrum of pharmacological activities, *i.e.,* antipyretic, hypertensive, antiurolithiasis, antisteroidogenic, antimicrobial, antiurolithic, anti-inflammatory, and central nervous system depressant activities (Loganayaki and Manian 2012). Furthermore, it also shows cytotoxic activity against human gastric, colon, cervical, and breast cancer cell lines [19-20], The use *of Clinacanthus nutans*, a small shrub native to tropical Asia, as herbal medicinal plant has long been used in Thai folk medicine for the treatment of skin rashes, insect and snake bites, inflammation, and herpes simplex and varicella zoster virus lesions (Janwitayanuchit et al., 2003).

The immune-stimulating effect of the remedy on NK cell activity was observed *in vitro* using Yac-1 cells as susceptible target cells. The NK cell activity of the nude mice treated with daily dose of the remedy for 1 and 2 weeks was found to be significantly increased in comparison with the corresponding control groups. Histopathological finding supported the stimulatory effect of the remedy on the lymphoid follicles in the spleen of nude mice. These observations thus support the role of NK cells in producing anticancer activity of the remedy in the xenografted nude mice. The significant increase of NK cell activity in yac-1 cell lysis indicates either the increase in the number of the NK cells in splenic mononuclear cells, or in NK cell activity, or both. The role of NK cells as the first-line non-specific immune defense mechanism is well recognized (Welsh, 1978; Herberman and Ortaldo, 1982). Unlike MMC, the inhibitory activity of the remedy on tumor growth was however limited to only the early period of the treatment (days 14-28). This suggests that NK cells effectively inhibited the growth of the tumor only when they were in small size. Extensive tumor growth can overcome their inhibitory effect, making the situation unamendable to immunological control. This could be due to the loss of certain components of tumor cell surfaces, *e.g.,* NK cell receptors. In addition, tumor cells can also non-specifically modulate host immunity through the release of soluble factors that directly suppress immunological reactivity (Greenberg et al., 1987). Suppression of natural killer cell activity has been observed in the peripheral blood of patients with various types of advanced solid malignancies (Takasugi et al., 1977). Furthermore, NK cell activity is usually not observed in large but in small mammary carcinoma (Welsh, 1978). The increase in the body weight in mice treated with the extract is probably due to its effect on nutritional and/or immunological function.

Apart from the immuno-modulatory activity, another possible mechanism for the therapeutic effect of this remedy is possibly due to its action on the hormone regulation system by some certain mechanism. This is especially evident in such hormone-dependent cancer as mammary cancer, in which the cells from the target tissue are in critical need of the hormone to maintain their growth and differentiation. It is believed that this effect depends largely on the degree of their hormone dependency and therefore, the effect of hormone or hormonimimetic substance on tumor growth is greater during the early stage. In the last stage, only few cancers remain hormone responsive (Trichieri and Santoli, 1978). If the antitumor activity of the remedy is through direct cytotoxic activity, the activation of the parent compound(s) to active moiety (ies) by host and/or the dynamic environment of host-tumor is required. However, the remedy showed virtually no toxicity in normal rats and mice in the toxicity testing. To confirm that the anticancer activity of the remedy is due to the immune-enhancing effect, further study in mammary cancer patients should be performed. The major problem encountered with current cancer chemotherapy is the undesirable toxicity and immunosuppression and agents with immune-stimulating activity may be used as adjunct therapy with conventional chemotherapeutic drugs to maximize therapeutic efficacy and tolerability.

CONCLUSION

The remedy was shown to exert anticancer activity in two

animal models (mammary cancer in rats and cervical cancer xenografted nude mice) without significant cytotoxic activity in vitro. Its anticancer activity could be through immunostimulating action on NK cell activity.

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