

RESEARCH ARTICLE

Immunostimulating Effect of a Well-known Thai Folkloric Remedy in Breast Cancer Patients

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Abstract

The study aimed to evaluate immune-stimulating effects of a well-known Thai folkloric remedy when used for adjuvant therapy with conventional chemotherapeutics for treatment of breast cancer. Immunostimulating influence of the remedy (215 mg/kg body weight per day) on NK cell activity and TNF- α release from the monocytes/macrophages were investigated in a total of 15 healthy women and 13 female patients with breast cancer (Group 1). The effect of breast tumor surgery on NK cell activity was further investigated in 18 female patients with breast cancer (Group 2). NK cell cytotoxic activity was determined by chromium release cytotoxic assay using K562, an erythroleukemic cell line. TNF- α release from monocytes/macrophages separated from blood samples was determined through a biological assay using actinomycin D-treated L929 mouse fibroblast cells in the presence and absence of LPS. Baseline NK cell activity of the monocytes/macrophages separated from Group 2 patients expressed as % cytotoxicity was significantly lower than in the healthy subjects at E:T ratios of 100:1 and 25:1. In healthy subjects, there was no change in NK cell cytotoxic activity (% cytotoxicity or LU) following 1 and 2 weeks of treatment with the remedy compared with the baseline at various E:T ratios but the binding activity (% binding) was significantly increased after 2 weeks of treatment. The addition of one or two conventional chemotherapeutic regimens did not significantly reduce the NK cytotoxic activity but did affect release of TNF- α in both unstimulated and LPS-stimulated samples. Surgery produced a significant suppressive effect on NK cell activity. The use of the remedy as an adjunct therapy may improve therapeutic efficacy and safety profiles of conventional chemotherapeutic regimens through stimulation of the immune system in cancer patients.

Keywords: Thai folkloric remedy - breast cancer - NK cells - immunostimulatory influence

Asian Pacific J Cancer Prev, 14 (4), 2599-2605

Introduction

Breast cancer ranks as the most common form of female malignancy in several geographical areas of the world. Statistics in 2010 showed the highest incidence of breast cancer compared to other types of cancer among women in Thailand (National Institute, 2012).

The major approaches currently being used in the management of this type of cancer include surgery, radiation, and chemotherapy. These therapeutic approaches are however unimpressive, due mainly to their toxicity, immune-suppression, mutagenicity, carcinogenicity, and drug resistance. Furthermore, most of these approaches are unaffordable to patients from developing countries. A large number of cancer patients therefore, usually seek alternative treatments which they find more effective with fewer side effects and also less expensive. Traditional

medicine has been recognized as an alternative treatment approach for cancer patients, particularly those with advanced stage cancer who do not find hope in treatment through modern medicine. Very commonly, cancer patients are treated with a mixture of herbs or herbal decoction, concurrently with some form of ritualistic ceremony and incantation.

One of the most well-known Thai folkloric remedies for treatment of various types of cancer which consists of a mixture of various parts from five plant, five animal species, and an artifact has been prescribed to cancer patients by traditional medicine practitioner Dr. Sommai Thongprasert for more than 40 years. The remedy has been used as an alternative treatment or adjuvant therapy depending on the type and severity of malignancy, pathological stage, and physical condition of the patients. It is claimed to be well-tolerated and exceptionally

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effective for the treatment of breast cancer when used as adjuvant therapy with conventional chemotherapeutic agents. The use of this remedy is claimed to significantly prolong life, reduce suffering, and improve appetite and body weight that result in the promotion of quality of life. Its reputation spreads to cancer patients in the neighboring countries such as Singapore and Malaysia. A preliminary study *in vitro* and in animal models demonstrated the promising anticancer and immunostimulating activities of this remedy without cytotoxicity *in vitro* (Na-Bangchang et al., 2012). The aim of this study was to evaluate the immunostimulating effect of this remedy when used as an adjuvant therapy with conventional chemotherapeutics for the treatment of breast cancer patients.

Materials and Methods

Chemicals and reagents

Commercial grade methanol and hydrochloric acid were purchased from RCI Labscan Co. Ltd. (Pathumwan, BKK, THA). Eagle's Minimum Essential Medium (EMEM), Hank Balance Salt Solution (HBSS), TNF- α , and fetal bovine albumin were purchased from Gibco BRL Life Technologies (Grand Island, NY, USA). Penicillin and streptomycin were purchased from Dumex (Bangkok, BKK, THA).

Preparation of plant extracts

One set of the remedy consists of a mixture of parts from five plants, five animals, and an artifact as follows: (a) Plants: *Polygala chinensis* (Ya Peek Kai Dam, Polygalaceae family, 6.0%, whole part), *Ammania baccifera* (Fai Duen Haa, Lythraceae family, 6.0%, whole part), *Clinacanthus nutans* (Phaya Yor or Phaya Pling Thong, Acanthaceae family, 3.5%, stems and leaves), *Canna indica* (Puttaraksa, Cannaceae family, 60.0%, rhizomes), *Smilax corbularia* (Khao-Yen-Nuea, Smilacaceae family, 7.0%, rhizomes), and (b) Animals: *Manis javanica* (Nim, 5.0%, scales), *Hystrix brachyuran* (Menn, 5.0%, spines), *Damonia subtrijuga* (Tao Naa, 9.5%, carapaces), *Dasyatis* spp. (1.5%, spines), *Trionyx cartilagineus* (Ta Pabb Nam, 3.5%, sternums), and (c) an artifact (a small piece 6x6 cm² of bamboo bucket used for holding water called "Kru").

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°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 xg 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°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 Equipment Gardiner Co. Ltd., NY, USA). The dark brown lyophilized remedy was ground into powder and stored at 4°C 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.

Human subjects and study design

Approval of the study protocol was obtained from the Ethics Committee of Mahidol University, Bangkok, Thailand.

Healthy subjects: A total of 15 healthy female subjects aged 30-43 yrs old, weighing 45-55 kg, who had no history of breast diseases, diabetes mellitus, hypertension, and allergic diseases were included in the study. All had no history of previous drug administration for at least two weeks before and during the study period. The investigation of the effect of the remedy on NK cell activity was performed on 8 subjects. Heparinized blood (10 ml) was collected from all of them via venipuncture once a week before breakfast for three consecutive weeks to establish the baseline NK cell activity status. Administration of the first dose of the remedy (a daily dose of 215 mg/kg body weight for two weeks) started after the last blood collection on week 3. All volunteers were healthy as verified by physical examinations and laboratory tests (hematology and biochemistry).

Patients with breast cancer: The study was conducted on two groups of breast cancer patients (31 females). The diagnosis of breast cancer in both groups was through clinical examination and/or ultrasonography.

Group 1 patients (13 females, aged 32-60 yrs old) received treatment with the remedy in combination with conventional chemotherapeutics at the Dr. Sommai Cancer Clinic, Singburi Province, Thailand. Five cases had previously been confirmed with the diagnosis of mammary cancer (adenocarcinoma) by histopathological examination of the biopsy specimen, 7 cases had previously undergone definitive clinical diagnosis of breast cancer (tumor diameter 1.5- 4.0 cm) and later on confirmation of adenocarcinoma was done by histopathology, and 1 case had previously received treatment with modified radical mastectomy together with 6 cycles of adjuvant chemotherapy (Table 1). Treatment regimen for patients in this group consisted of the remedy (a daily dose of 215 mg/kg body weight for 24 weeks) in combination with conventional chemotherapeutics (cyclophosphamide, tamoxifen, mitomycin C-2, 5-fluorouracil, and prednisolone) as summarized in Table 2.

Group 2 patients (18 females, aged 35-68 years old) underwent modified radical mastectomy with auxiliary lymph node dissection, followed by adjunct treatment with conventional chemotherapeutics (FAC: 5-fluorouracil,

Table 1. Characteristics of Group 1 Patients (13 cases) who Received Treatment at Dr. Sommai Cancer Clinic, Singhaburi Province, Thailand

Patient No.	Age (years)	Clinical description of mammary cancer
1	32	Post-biopsy with residual tumor (1 cm in diameter)
2	35	Post-biopsy
3	39	Post-biopsy
4	48	Post-biopsy
5	60	Post-biopsy
6	42	Tumor (3 cm in diameter) with nipple retraction, confirmed breast cancer (adenocarcinoma) by tissue biopsy after treatment.
7	49	Tumor (4 cm in diameter) with nipple retraction, confirmed breast cancer (adenocarcinoma) by tissue biopsy after treatment.
8	34	Tumor (2.5 cm in diameter) with nipple retraction, confirmed breast cancer (adenocarcinoma) by tissue biopsy after treatment.
9	42	Tumor (3 cm in diameter) with nipple retraction, confirmed breast cancer (adenocarcinoma) by tissue biopsy after treatment.
10	32	Tumor (1.5 cm in diameter) with irregular surface and fixed to chest wall, confirmed breast cancer (adenocarcinoma) by tissue biopsy after treatment.
11	32	Tumor (2 cm in diameter) with irregular surface and fixed to chest wall, confirmed breast cancer (adenocarcinoma) by tissue biopsy after treatment.
12	35	Tumor (2 cm in diameter) with irregular surface and fixed to chest wall, confirmed breast cancer (adenocarcinoma) by tissue biopsy after treatment.
13	48	Post-NRM and chemotherapy of 6 cycle with palpable lymph node
Median (range)	36 (32-60)	

Table 2. Treatment Regimen in Patients with Breast Cancer in Group 1. The Folkloric Remedy was Given at a Dose of 500 ml Per day for 24 Weeks (6 months)

Day of treatment	Dose regimen/Blood collection
Day 14 (Week 2)	Blood sample collection Folk remedy+Start and continue A
Day 28 (Week 4)	Blood sample collection Folk remedy+Continue A+Start B
Day 42 (Week 6)	Blood sample collection Folk remedy+Continue A+B
Week 6-24	Blood sample collection Folk remedy

*A=50 mg (bid., po, daily) Cyclophosphamide+10 mg (bid., po., daily) Tamoxifen. B=500 mg (single, iv) 5-Fluorouracil+2 mg (single, iv.) Mitomycin C-2+200 mg (single, i.v.)+Cyclophosphamide in 5% dextrose in 1000 ml NSS/2 dripped in 4-6 hr+100 mg (single, im.) Testosterone+5 mg (single, im.) Prednisolone

doxorubicin, and cyclophosphamide given 3-weekly for 6 cycles) at the Ramathibordi Hospital, Bangkok, Thailand. Inclusion criteria included \geq stage II infiltrating breast adenocarcinoma, with no previous or concurrent cancer of other organs, diabetes mellitus, hypertension, allergic diseases, and with no previous treatment with radiation, chemotherapeutics or hormones.

Prior to the treatment, full physical and clinical examination were performed on patients of both groups. Heparinized blood samples (10 ml each) were collected from all patients before the administration of each treatment period for the assessment of NK cell cytotoxicity and TNF- α release activity (only Group 1). In addition,

chest x-ray and laboratory investigation (hematology, biochemistry, and urinalysis) were performed on Group 2 patients.

Assessment of the effect on NK cell activity

Baseline NK cell activity of the effector NK cells (plastic non-adherence cells) was investigated in all healthy women (15 cases) and patients with breast cancer (31 cases) using the standard chromium-release cytotoxic assay (Pross et al., 1981) with modifications. The effect of the remedy when used alone or in combination with conventional chemotherapeutics on NK cell cytotoxic activity was investigated only in 12, 5, and 3 patients from Group 2 following treatment with the remedy alone, remedy in combination with one (A), and two (A+B) conventional chemotherapeutic regimens, respectively. Due to limitation of blood sample quantity, the effect of surgery on NK cell activity was accomplished in only 4 patients from Group 2.

Preparation of effector cells: Mononuclear cells were separated from peripheral blood samples (Boyum, 1968) with slight modifications under aseptic condition. In brief, the mononuclear cell suspension was allowed to adhere to plastic petri-dish at a density of 2×10^5 cells/cm² for 1 h in a humidified 37°C incubator with 5%CO₂. Non-adherent cells were collected through centrifugation (400 xg for 10 min). Cell pellets were resuspended in a RPMI 1640 medium and cell number was adjusted to 1×10^7 cells/ml. The viability of effector cells population as determined by trypan blue exclusion was $>95\%$. The adherent cells were removed from the surface of the dishes by incubation with a RPMI 1640 medium supplemented with 10% heat-activated fetal bovine serum and 5 mM EDTA (RPMI 1640 complete medium) at 37°C in 5%CO₂ incubator for 30 min. Cells were separated through centrifugation at 800 xg for 5 min. Cell pellets were resuspended in a RPMI 1640 complete medium and cell number was adjusted to 5×10^5 cells/ml. These cells were identified by esterase staining as monocytes/macrophages.

NK cell cytotoxicity assay: K562, an erythroleukemic cell line derived from a pleural effusion of a patient with chronic myelocytic leukemia in blast crisis (Lozzio and Lozzio, 1977), was used as the target cell for NK cytotoxic assay. The cells were maintained in a RPMI 1640 complete medium. Viability of the cells was checked by trypan blue dye exclusion. The assay was performed in triplicates in a standard 96-well round-bottom tissue culture plates (Nunc, Intermed Co. Ltd., Copenhagen, Denmark) in a total volume of 200 μ l. ⁵¹Cr-labeled K562 cells (1×10^4 cells/100 μ l) were added into each well and the effector: target (E:T) cell ratios of 100:1, 50:1, 25:1, and 12.5: 1 were prepared. Control wells for measuring spontaneous chromium release contained 100 μ l of 1×10^4 target cells and 100 μ l of a RPMI 1640 complete medium. Maximum releasable chromium was determined by adding 100 μ l of 1×10^4 ⁵¹Cr-labelled target cells to 100 μ l of Triton X-100. ⁵¹Cr released from spontaneous and maximum release wells were simultaneously determined in each experiment. The plates were centrifuged at 50 xg for 5 min and incubated at 37°C in a humidified 5%CO₂ incubator for 4 h. At the end of the incubation period,

cell supernatant in each well was separated through the centrifugation of the plates at 50 xg (4°C) for 10 min. An aliquot of 100 µl of supernatant was collected into a plastic tube and radioactivity was measured using a gamma counter (1260 Multigamma II. LKB, Stockholm, Sweden). NK cell cytotoxic activity was expressed as % cytotoxicity and lytic unit (LU: the number of effector cells required to cause 50% lysis of target cell) per 10⁷ cells (Cerottini and Brenner, 1974). The cytotoxic activity was calculated according to the formula: %Cytotoxic activity = [(Experimental release - Spontaneous release) x 100/ (Maximum release - Spontaneous release)]

Experimental release is the mean count *per minute* (*cpm*) of supernatant from the quadruplicate wells containing a mixture of effector cells 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.

Assessment of the stimulatory effect on TNF- α release

L929, a transformed cell line originally derived from the C3H strain of mouse fibroblast was used as the target cell for the measurement of TNF- α activity of the monocytes/macrophages separated from 6 healthy subjects and 7 breast cancer patients in Group 1 before and after treatment with the remedy and remedy in combination with therapeutic regimen A. The cells were grown as an adherent monolayer in a RPMI 1640 in a sterile plastic disposable tissue culture flask (75 ml, Nunc, Copenhagen, Denmark) and sub-cultured every 3 days. TNF- α released from the cells was determined by a biological assay using actinomycin D-treated L929 mouse fibroblast cells in the presence (activated) and absence (non-activated) of LPS (*Escherichia coli* 0111: B4) for 24 h (Ruff and Gifford, 1980). The assay was performed in triplicates. Briefly, L929 cells (3×10^4 in 100 µl RPMI 1940 medium) were seeded into 96-well flat-bottom tissue culture plates and allowed to grow for 24 h. On the day of the assay, the culture medium was discarded and two-fold serial dilutions of the culture supernatant, recombinant TNF- α , and 1 µg/ml actinomycin D were added (final volume 200 µl/well). The control wells contained L929 cells culture supernatant and 1 µg/ml of actinomycin D. Following 18 h of incubation (37°C, in a humidified 5%CO₂), the fluid in each well was discarded and the monolayer cells were washed three times with PBS to remove dead and non-adherent cells. The remaining cells were stained with 0.5% crystal violet in 25% methanol, washed with distilled water, and solubilized with 100 µl of acidified methanol (100 mM in hydrochloric acid). The absorbance was measured at 540 nm using a multi-scanning ELISA reader (Titertek Multoskan MCC/340 MK II, Flow Laboratories, Geneva, Switzerland). Percent cytotoxic activity was used to indicate TNF- α released from the cells according to the formula: Cytotoxicity (%) = [(1-OD₅₄₀ Experiment) x 100/ OD₅₄₀ Control].

Statistical analysis

All quantitative variables are presented as median

(range). Comparison of all quantitative variables between the two groups was performed using Mann-Whitney U test and between pre- and post-treatment was by Wilcoxon signed rank test (SPSS version 11). Statistical significance level was set at $\alpha=0.05$ for all tests.

Results

Effect of the remedy on NK cell activity

Baseline NK cell activity: NK cell activity measured by standard chromium-release cytotoxic assay of healthy women (15 cases) and patients with breast cancer in Group 1 (13 cases) and Group 2 (18 cases) before treatment is shown in Table 3. The cytotoxic activity of the target K562 cells was decreased by increasing the dilution of the effector cells from 100:1 to 12.5:1 in all groups. The NK cell cytotoxic activity expressed as either % cytotoxicity or lytic unit (LU) of the monocytes/macrophages separated from Group 1 breast cancer patients was similar to that of the healthy subjects at all E:T ratios. The NK cell activity of the monocytes/macrophages separated from Group 2 patients expressed as % cytotoxicity was significantly lower than the healthy subjects at the E:T ratios of 100:1 and 25:1.

Effect of the remedy alone and in combination with conventional chemotherapeutics on NK cell activity

Healthy subjects: The cytotoxic activity of the monocytes/macrophages separated from the blood of healthy subjects following treatment with the remedy for 1 or 2 weeks was decreased with the increasing dilution of the effector cells from 100:1 to 12.5:1. There was no change in NK cell cytotoxic activity (expressed as either % cytotoxicity or LU) following 1 and 2 weeks of treatment with the remedy and compared with the baseline at various

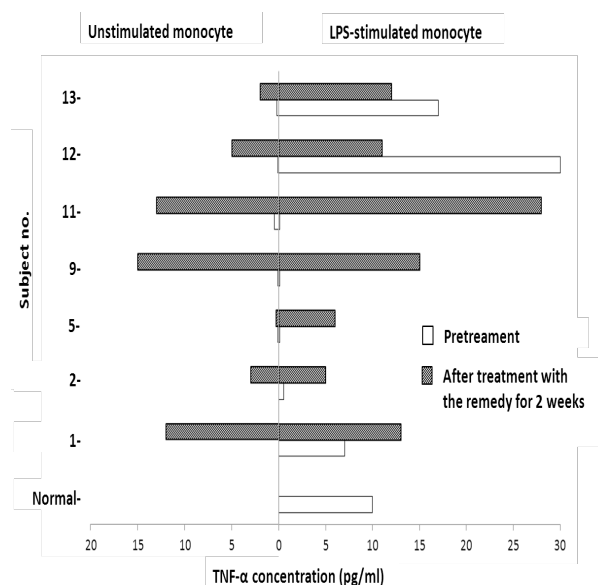


Figure 1. TNF- α Levels from the Monocytes/ Macrophages (unstimulated- and LPS-stimulated) of 7 Patients with Breast Cancer in Group 1 before and after the Treatment with the Remedy for 2 Weeks (215 mg/kg body weight per day)

E:T ratios was significantly increased after 2 weeks of treatment with the remedy (Table 3). All volunteers were healthy as verified by physical examinations and laboratory tests performed on each subject before each treatment period.

Mammary cancer patients: The NK cell cytotoxic activity of the monocytes/macrophages separated from the blood of Group 1 patients with breast cancer was investigated after 2 weeks of treatment with the extract alone (12 cases) or when given in combination with one (chemotherapeutic regimen A: 5 cases) or two (chemotherapeutic regimen B: 3 cases) conventional chemotherapeutic regimens. The lytic unit and cytotoxic activity (%) at the E:T ratios of 100:1, 50:1, and 25:1:1 was significantly increased after 2 weeks of treatment with the remedy alone compared with the pre-treatment level. The decline in the activity when one or two conventional chemotherapeutic regimens was added was not statistically significant (Table 3).

Effect of surgery on NK cell activity

The effect of surgery on NK cell activity was also investigated in 4 patients in Group 2. Surgery produced a significant suppressive effect on the host immunity, with a significant reduction of cytotoxic activity (% cytotoxicity at almost all E:T ratios, and lytic unit) (Table 3).

Effect of the remedy on TNF- α release in healthy subjects

The specificity of the assay method was demonstrated by the detection of TNF- α in both culture supernatant of unstimulated and LPS-stimulated monocytes/macrophages but not in the culture supernatant of lymphocytes either with or without LPS stimulation. Cultured monocytes spontaneously released modest levels of TNF- α when

cultured with 10 ng/ml LPS. Median (range) TNF- α level released from LPS-stimulated monocytes from 6 healthy subjects was 10.40 (0.40-18.98) pg/ml.

TNF- α released by monocytes/macrophages isolated from blood of patients with breast cancer in response to treatment

TNF- α released from the monocytes/macrophages (LPS-stimulated and unstimulated) in response to the treatment was investigated in 6 healthy subjects and 7 patients with breast cancer in Group 1 before and after the treatment with the remedy and the remedy in combination with chemotherapeutic regimen A.

Healthy subjects: TNF- α activity was detected in both culture supernatant of unstimulated and LPS-stimulated monocytes but not in the culture supernatant of lymphocytes either with or without LPS stimulation indicating specificity of the assay. Cultured monocytes spontaneously released modest levels of TNF- α but with a significant level following stimulation with 10 ng/ml LPS [median (range) = 10.40 (9.02-12.04) pg/ml].

Breast cancer patients: TNF- α released from the unstimulated monocytes was undetectable in 4 subjects (No. 1, 5, 9, and 11). The levels in the other three cases were 0.180, 0.196, and 0.226 pg/ml. Following treatment with the remedy for 2 weeks, the level was increased in all of the seven cases [median (range) = 4.4 (0.48-1.80) pg/ml]. The addition of chemotherapeutics to the treatment schedule decreased the release of TNF- α in 2 cases whose monocytes/macrophages were available for the assay (0.440 and < 0.124 pg/ml) (Figure 1). The addition of LPS stimulated the release of TNF- α in five cases including those previously inactive to LPS-stimulation at pre-treatment (No. 5 and 9) compared to the unstimulated

Table 3. NK Cell Activity as Indicated by Cytotoxicity (% cytotoxicity at various E:T ratios and lytic unit) of the Monocytes/Macrophages Isolated from Blood Samples Collected from Healthy Female Subjects, Group 1 (Dr. Sommai Cancer Clinic, Singburi Province, Thailand) and Group 2 (Ramathibordi Hospital, Bangkok, Thailand) Mammary Cancer Patients at Baseline, and Following Treatment with the Remedy Alone, or in Combination with Chemotherapeutic Regimen A and B. Data are Presented as Median (range)

	Healthy Subjects (n=15)				Breast Cancer Patients						
	Baseline (n=15)	Pre -treatment week (n=15)	1 week (n=15)	2 week (n=15)	Group 1 (n=13)			Group 2 (n=18)			
					Baseline (n=13)	Pre -treatment	Remedy alone for 2 weeks (n=12)	Remedy + Chemotherapeutic regimen A (n=5)	Remedy + Chemotherapeutic regimen A+B (n=3)	Baseline (n=18)	Post -Surgery (n=4)
%Cytotoxicity:											
100:1	74.47 (71.13-46.02)	74.88 (72.55-76.58)	73.38 (71.88-75.69)	73.13 (70.56-75.02)	72.23 (69.05-75.44)	52.5 (50.21-55.78)	76.42* (73.60-80.41)	71.2 (66.95-72.12)	67.67 (66.35-72.15)	69.89** (66.51-71.11)	70.75 (66.33-71.19)
50:1	62.67 (60.63-64.25)	63.75 (60.34-65.02)	64.75 (61.08-67.89)	64.38 (62.67-68.04)	59.62 (56.97-62.11)	59.75 (53.22-62.99)	66.92* (63.22-71.02)	56.17 (53.34-57.78)	54.33 (50.35-56.19)	58.5 (56.01-60.25)	55.50*** (53.12-56.12)
25:1	46 (45.49-47.02)	47.25 (45.01-49.02)	49.25 (45.59-53.78)	50 (45.23-53.64)	44.23 (42.49-45.12)	44.58 (41.55-68-47.02)	51.83* (48.47-52.14)	42.17 (37.28-43.01)	39 (35.50-42.05)	41.11** (40.11-42.18)	35.50*** (34.41-39.02)
12.5:1	26.4 (24.16-28.23)	-	-	-	28.33 (25.23-30.18)	39.67 (33.65-42.12)	35.33 (33.63-26.99)	23 (19.49-25.02)	29 (71.00-31.14)	26.47 (24.08-26.99)	19.50*** (17.48-23.36)
Lytic unit (LU/10 ⁷ cells)	34.14 (31.98-36.05)	35.79 (32.98-37.66)	42.71 (40.98-44.78)	36.75 (33.80-40.14)	32.26 (30.60-35.67)	32.66 (30.89-35.47)	43.18 * (40.71-46.12)	32.65 (28.05-34.06)	29.62 (28.04-33.25)	30.56 (27.59-33.28)	25.38*** (23.39-30.15)

Statistically significant difference compared with baseline *p<0.01, Wilcoxon Signed Rank test. **p<0.01, Mann-Whitney U test. ***p<0.01, Wilcoxon Signed Rank test. ****p<0.05, Wilcoxon Signed Rank test

monocytes [median (range) = 12.0 (4.40-28.80) pg/ml] (Figure 1). The other two cases (No. 12 and 13) whose monocytes had previously been highly active to LPS-stimulation became less active following treatment with the remedy (10.80 vs. 30.00 and 12.00 vs. 17.20 pg/ml, respectively). The addition of one chemotherapeutic drug to the treatment schedule of the remedy decreased the release of TNF- α in both of the two cases (No. 2 and 9) whose blood samples were available for the assay (0.38 vs 4.40 and 1.08 vs. 15.20 pg/ml, respectively).

Discussion

This study initially investigated baseline NK cell function in the monocytes/macrophages of breast cancer patients in comparison with the healthy women. The NK cytotoxic activity was significantly lower in Group 2 patients (Ramathibordi Hospital) while the activity in Group 1 patients was similar compared with the healthy subjects. This may suggest the difference in severity of breast cancer between the two groups of patients. In addition, conditions of stress and depression in the first group of patients before tumor operation may have influenced the immune status of the patients in Group 2 (Irwin et al., 1987; Levy et al., 1987; Talmadge and Alvord, 1987). The lytic figures were similar in all these three groups. Following treatment with the remedy for 1 and 2 weeks, NK cell activity in healthy subjects remained unchanged. On the other hand, significant increase in the cytotoxic activity (both as % cytotoxicity and lytic unit) was observed in Group 1 patients after the treatment with the remedy for two weeks. Altogether, this suggests that tumor cell is one of the key factors that play a role in challenging NK cell activity. With this limited sample size, the decline in NK cell activity (cytotoxic and lytic activities) when the courses of the conventional chemotherapeutics (A, and A+ B) were added to the treatment schedule of the remedy was not statistically significant. Direct comparative investigation (in two independent groups following the treatment with the remedy alone and in combination with chemotherapeutics) on the effect of chemotherapeutic alone and in combination with the remedy was not performed due to ethical concerns. Several medicinal plant extracts are currently receiving great attention for their enhancing effects on the non-specific immune response in cancer patients (Burana-Osot et al., 2010; Salagianni et al., 2012). Stimulation of NK cell activity was demonstrated with *Astragalus oxyphyllus* (McCaleb, 1989), *Glycyrrhiza glabra* L. (Abe et al., 1982), and *Viscum album* (Wagner, 1985). Stimulation of T-cell activity and synergistic effect with IL-2 was shown with the polysaccharide fraction isolated from *Astragalus membranaceus* (Sun et al., 1983). In addition, induction of interferon production was reported with the polysaccharide fraction isolated from the bark of *Angelica acutiloba* induced interferon production (Yamada et al., 1984).

The response of NK cells to various stimuli is divided into three sequential phases (Salagianni et al., 2012). The first phase was a rapid response (1-10 min) associated with cancer cytotoxicity and the release of granule contents.

The second phase (10-120 min) involves the transcription and expression of genes encoding lymphokines and surface activation antigens. The third phase (1-3 days) of the response was blast formation, DNA synthesis, and proliferation. Various stimuli or modulating factors affected these three phases of the NK cell response. The observed stimulatory activity of the NK cell activity in breast cancer patients in the present study after treatment with the remedy could be a consequence of an increase in NK cell number directly involved in NK cell cytotoxic activity in peripheral blood lymphocyte (PBL) population (centralized proliferation of NK progenitor cells in bone marrow or the induction of circulating NK cells into cell cycle) or an activation of the cytokine machinery (activation of the expression of adhesion molecules on NK cell surface that bind to the target cells or an increase in catalytic granules in the NK cells) or an activation of NK cells to highly active state. The first assumption can be confirmed by morphological or phenotypic analysis of NK cell population. The surface molecule which might play important functional roles in NK cell cytotoxicity include CD11/CD18 family, laminin and its receptor, and CD2 antigen (Hiserodt et al., 1982; Schmidt et al., 1985; Salagianni et al., 2012). The release of cytokines by NK cells or other effector cells in the immune system or cytokine receptors on NK cell surface following stimulation by the remedy in turn activates NK cell activities. Among the cytokines generated by NK cells, IL-2, IFNs, and TNF- α activates other immune cells that are responsible for cancer cell cytotoxicity (Salagianni et al., 2012).

The current chemotherapeutic modalities for the treatment of breast cancer are the adjunct of chemotherapy immediately after surgery. This present study showed a significant reduction in NK cell in all 4 cases after breast tumor operation (before the treatment with chemotherapeutic regimen). This finding supports previous reports on the reduction of NK cell activity in breast cancer patients following surgery (Pollock and Lotzova, 1987). This suppression in NK cell activity would in turn facilitates tumor metastasis. Supplement therapy with this folk remedy may prove beneficial to patients who receive chemotherapeutics and/or surgery since it promotes the immune effector function.

TNF- α , the cytokine produced by the human host, is a molecule that is involved in tumor cell killing as well as in a wide variety of biological activities (Locksley et al., 2001). Results of the current preliminary study suggest the potential of the extract in stimulating the release of TNF- α from the monocytes/macrophages. Restoration of TNF- α release after the treatment with the remedy was also observed in all of the seven patients who had relatively low release in response to LPS stimulation at baseline. It was noted that 5 patients (No. 1, 9, 11, 12, and 13) whose tumor cells were still present at the time of blood collection for TNF- α release analysis, showed good activity of monocytes/macrophages in response to both LPS and treatment with the remedy. The addition of chemotherapeutic regimen A appears to decrease the release of TNF- α . Unfortunately this could be confirmed only by two cases whose blood samples were available

for the assay. Results suggest that the presence of tumor cells can activate the response of monocyte/macrophage stimulation. A variety of immunological approaches for treating cancers including antitumor antibodies, adoptive cellular immunotherapy, and cytokines treatment are currently in clinical trials (Monjazeb et al., 2013). Several of these exogenous approaches are highly toxic at the doses required for tumor killing. The remedy enhanced both endogenous NK cell and monocyte/macrophage activity in the same manner as exogenous stimulating substances but without significant adverse effects during the course of the treatment. All patients had better tolerability to the chemotherapeutic courses in the presence of the remedy. Further studies in a larger sample size should be conducted to confirm the therapeutic efficacy and safety of the extract when used as adjuvant therapy with conventional chemotherapeutics and surgery. The use of the remedy as an adjunct therapy may improve therapeutic efficacy and safety profiles of conventional chemotherapeutic regimens through the stimulation immune status of cancer patients. Further study in a larger number of patients is required.

Acknowledgements

The study was supported by the China Medical Board, Siriraj Hospital and the Faculty of Graduate Studies, Mahidol University, Bangkok, Thailand.

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