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Effect of Clinacanthus nutans on Human Cell-mediated Immune Response In Vitro(การศึกษาฤทธิ์ของสารสกัดจากพญายอต่อการตอบสนองทางภูมิคุ้มกัน...

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Effect of *Clinacanthus nutans* on Human Cell-mediated Immune Response *In Vitro*

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ABSTRACT: The extract of *Clinacanthus nutans* has been used for the treatment against a variety of symptoms from either infectious or non-infectious agents. Less has been known for its activity on the immune system. Human immunocompetent cells were used to study the role of *C. nutans* extract in modulating cell-mediated immune response (CMIR) *in vitro*. We investigated the effect of *C. nutans* extract on lymphocyte proliferation, function of natural killer (NK) cells, and production of interleukin-2 (IL-2) and interleukin-4 (IL-4). Lymphocyte proliferation was significantly increased at the concentrations of 0.5, 2.5, and 5 μg/ml, while the response was significantly reduced at 2.5, and 5 mg/ml of *C. nutans* extract. The activity of NK cells was significantly decreased at the concentrations of 1 and 5 mg/ml. Furthermore the level of IL-2 released from *C. nutans* treated-mononuclear cells was undetectable, whereas that of IL-4 was shown to be induced by the extract at the concentrations of 2.5 and 5 mg/ml. These data suggested that the effect of *C. nutans* on human CMIR *in vitro* may be partially due to the release of IL-4 from peripheral blood mononuclear cells.

KEY WORDS: *Clinacanthus nutans,* CMIR

INTRODUCTION

In traditional medicine, fresh leaves of *Clinacanthus nutans* (Burm. f.) Lindau has been suggested to be effective in the treatment of poisonous snake and insect bites, burns, and allergic reactions (1). Alcoholic extract of the leaves of this plant significantly reduced the lethal effect of cobra venom administered intravenously (2). Further study in pharmacological properties of *C. nutans* revealed that butanol extract exhibited anti-inflammatory action in rats receiving carrageenan (3).

The extract of *C. nutans* was recently shown to inactivate herpes simplex virus type 2 (HSV-2) and varicella zoster virus (VZV) *in vitro* (4, 5) and demonstrated good efficacy in shortening the duration of HSV-2 infection and reduction in its severity in clinical trial studies (6). In addition, ethanol extract of *C. nutans* was shown to inhibit the growth of yellow head baculovirus (YBV) and also induce phagocytosis activity in tiger prawn (7).

These lines of evidence suggested that traditional medicine can be very valuable and useful for modern scientific studies. The therapeutic effects of *C. nutans* extract may be due to an influence on the immune system; therefore, the immunomodulatory capabilities of *C. nutans* were investigated for a better understanding of its mechanisms of actions. In the present study, we examined the abilities of *C. nutans* extract on cell-mediated immune response (CMIR) by studying its effects on lymphocyte proliferation, natural killer (NK) cell activity and cytokine production of human peripheral blood mononuclear cells (PBMCs)

MATERIALS AND METHODS

Preparation of *C. nutans* extract

Dried leaves were extracted with ethanol in a soxhlet apparatus and the extract was dried under vacuum in a rotary evaporator giving the yield of 15%. The extract was then mixed with polyvinyl pyrrolidine (PVP) at the ratio of 1:4

to form a water-soluble complex. The complex was weighed out to give a concentration needed and then dissolved in phosphate buffer saline (PBS) pH 7.2 for further studies.

Subjects

Twenty-six healthy Thai adults were recruited in this study. None had a history of hepatitis B infection, and exposed to human immunodeficiency virus type 1. None were taking antimalarials, corticosteroids or immunosuppressive drugs.

Preparation of mononuclear cells

Heparinized peripheral blood was obtained aseptically from those subjects. Mononuclear cells were separated from the heparinized blood using Ficoll-Hypaque density gradient (8). Two parts of heparinized blood were gently layer on three parts of Ficoll-Hypaque mixture (IsoPrep, Robbins Scientific Corporation, Sunnyvale, CA, USA) and spinned at 400 g for 30 minutes at 25 °C. The mononuclear cells were collected from the white band at the interface of the gradient and plasma, and washed 3 times with RPMI 1640 (Grand Island Biological Company, Grand Island, NY, USA) by centrifugation at 400 g for 10 minutes at 4 °C. The mononuclear cells were counted and adjusted to an appropriate concentration in complete RPMI 1640 (RPMI 1640 medium supplemented with 2 mM glutamine, 10 mM HEPES, 100 U/ml penicillin G, and 100 μg/ml streptomycin) containing 10% fetal bovine serum (FBS; Grand Island Biological Company, Grnad Island, NY, USA) for further assays.

Lymphocyte proliferation response assay

Purified mononuclear cells (2×10^6 cells/ml) were cultured in triplicate in 96-well microtiter plates (Costar, Cambridge, MA, USA) with *C. nutans* extract at the concentrations of 1, 5, 10, 100, 1000, 2500, 5000, and 10000 μg/ml to give final concentrations of 0.5, 2.5, 5, 50, 500 μg/ml, 1.25, 2.5 and 5 mg/ml, respectively, in complete RPMI 1640 containing 10% FBS. The cultures were incubated at 37 °C with 5% CO_2 for 72 hours. Eighteen hours before harvest, 20 μl of 0.5 μCi ^{3}H-thymidine (specific activity 8.3 mCi/mg: Amersham, Buckinghamshire, UK) was added. ^{3}H-thymidine incorporation was determined by harvesting with a multichannel automatic cell harvester (CH-103, Dynatech Lab, Inc., Sussex, UK) onto glass fiber filters (Whatman 934 AH, Whatman International Ltd., Maidstone, England). The radioactivity was measured by a liquid scintillation counter (1211/1212 Rackbeta liquid scintillation counter, Wallac Oy, Turku, Finland). The degree of activation was expressed as a stimulation index [S.I., i.e., the ratio of the ^{3}H-thymidine uptake in count per minute (CPM) of samples with extract to those without extract]. Phytohemagglutinin HA16/17 (Murex Diagnostics Limited, Dartford, England) at 2 μg/ml was also added to the culture system to serve as positive control.

NK cell activity assay

Peripheral blood mononuclear cells (PBMCs) were washed and resuspended in complete RPMI 1640. K 562 cells (gift of Dr. Molvibha Vongsakul, Faculty of Science, Mahidol University, Thailand) were used as target cells and were grown in complete RPMI 1640 containing 10% FBS. The target cells (1.5×10^6 cells) were labelled with $Na_2{}^{51}CrO_4$ (specific activity 37.0 MBq/ μg: Amersham, Buckinghamshire, UK) at 37 °C 5% CO_2 for 45 minutes, washed 3 times with cold RPMI 1640 containing 10% FBS.

The cytotoxicity assay was performed in round-bottom microtiter plates (Corning Glass Works, Corning, NY, USA) using 1×10^4 target cells/well and a PBMC effector-to-target cell ratios (E:T) of 100:1, 50:1, 25:1, and 5:1. Each condition was set up in triplicate with and without *C. nutans* extract at the final concentrations of 1 mg/ml and 5 mg/ml in a 96-well plate. The plates were incubated for four hours at 37 °C with 5% CO_2. After incubation, the plates were centrifuged at 200 g for 5 minutes. Supernatants from each well (100 μl) were transferred into tubes and counted in a Gamma counter (RIASTAR QC Gamma Counting Systems, Packard Instrumental Co., CT, USA). The percentage of cytolysis was calculated according to the following formula: % cytolysis = (experimental release - spontaneous release) / (maximal release - spontaneous release). Spontaneous release was measured by incubation of target cells with medium alone, while maximal release was measured by lysis of target cells with 5% Triton X-100. NK cell activity was expressed as lytic units (LU)/ 10^7 PBMCs as determined by least squares analysis derived from the percentage of specific lysis of all E:T ratios. One LU was defined as the number of effector cells required for 20% specific lysis of 1×10^4 target cells.

Flow cytometry analysis

Cell pellets, from the cultures containing the extract at the concentrations of 0.5, 2.5 μg/ml, 1, and 5 mg/ml, were washed with RPMI 1640 by centrifugation at 400 g for 5 minutes at room temperature. Cells were stained using CD3, CD3/CD4, CD3/CD8, CD3/CD16+56, and CD3/CD19 mouse monoclonal antibodies fluorescein conjugated (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) which were specific for CD3 (total T lymphocytes), CD4 (T helper/inducer cells), CD8 (T suppressor/cytotoxic cells), CD16/CD56 (NK cells), and CD19 (B lymphocytes) positive cells, respectively. The cells were incubated for 20 minutes at room temperature, washed in phosphate buffer saline (PBS) pH 7.2 by centrifugation at 300 g for 5 minutes. The cell pellets were fixed with 3% formaldehyde in PBS before analysis by flow cytometry in a Becton Dickinson FACScan system (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA).

Measurement of cytokine production

PBMCs (2×10^6 cells/ml) were cultured with *C. nutans* extract at the final concentrations of 0.5, 5, and 500 μg/ml and 1, 2.5, and 5 mg/ml. The cultures were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. After 24 hours of incubation, supernatants were harvested and levels of interleukin 2 (IL-2) and interleukin 4 (IL-4) were measured by enzyme linked immunosorbent assay (ELISA), according to the manufacturer's instruction (Genzyme, Cambridge, MA, USA).

Statistical analysis

Data were analyzed using paired t-test for lymphocyte proliferation test and for functional assay of NK cells.

RESULTS

Lymphocyte proliferation response

Similar patterns of lymphocyte responses were obtained in all normal volunteers. The reactivity was a dose-dependent response since there was no response without addition of the extracts (S.I. equals to 1). The lymphocyte proliferation was significantly elevated with *C. nutans* extracts ranging from concentrations of 0.5 μg/ml to 5 μg/ml ($P < 0.05$) (Table 1). No significant differences could be observed at the concentrations of 50 μg/ml, 500 μg/ml, and 1.25 mg/ml. At higher concentrations, i.e., 2.5 mg/ml and 5 mg/ml, the lymphocyte proliferation was significantly decreased as compared without the extract added in the same culture ($P < 0.05$) (Table 1).

Table 1 Effect on proliferation of peripheral blood mononuclear cells (PMBCs) by *C. nutans*

Treatment (extract concentrations)	Stimulation index (S.I.)[a] (n = 26)
none	1.00 ± 0.00
0.5 μg/ml	1.14 ± 0.25[b]
2.5 μg/ml	1.23 ± 0.48[b]
5 μg/ml	1.36 ± 0.68[b]
50 μg/ml	0.91 ± 0.35
500 μg/ml	0.88 ± 0.36
1.25 mg/ml	0.81 ± 0.49
2.5 mg/ml	0.75 ± 0.54[b]
5 mg/ml	0.69 ± 0.45[b]
PHA	100.45± 86.85

a. Each value represents mean ± SD (standard deviation)

b. P value < 0.05

NK activity

NK activity in the presence of *C. nutans* extract was assessed and compared with the one without the extract. A significant reduction in NK activity was demonstrated in PBMCs treated with 1 mg/ml of *C. nutans* extract as compared with untreated ones ($P < 0.05$). Additionally, NK activity was not detectable in PBMCs treated with *C. nutans* extract at a final concentration of 5 mg/ml (Table 2).

Table 2 Activity of cells with natural killer (NK) function in lytic units (LU)/10^7 peripheral blood mononuclear cells (PBMCs) in the presence of *C. nutans* extract.

Extract concentrations	LU/10^7 PBMCs[a] (n = 15)
none	103.10 ± 56.64
1mg/ml	42.04 ± 26.16[b]
5 mg/ml	0.01 ± 0.00[b]

a. mean ± SD

b. P value < 0.05

Effect of *C. nutans* extract on lymphocyte subpopulations

A profound reduction in NK activity was evident in specimens treated with 1 mg/ml and 5 mg/ml of *C. nutans* extract. We, therefore, examined whether the extract was able to alter the number of lymphocyte subsets. We randomly analyzed three samples by means of flow cytometer. Our results showed that there was no change in the percentages of $CD3^+$, $CD4^+$, $CD8^+$, $CD16^+/CD56^+$ and $CD19^+$ cells at all concentrations of *C. nutans* tested (Table 3).

Table 3 Effect of *C. nutans* on percentage of circulating $CD3^+$, $CD4^+$, $CD8^+$, $CD16^+/CD56^+$, and $CD19^+$ cells in three individuals.

Donor #	Treatment	$CD3^{+a}$	$CD4^{+a}$	$CD8^{+a}$	$CD16^+/CD56^{+a}$	$CD19^{+a}$
1	none	57	27	22	33	5
	0.5 μg/ml	52	22	20	35	4
	2.5 μg/ml	59	30	22	35	4
	1 mg/ml	62	32	25	38	5
	5 mg/ml	59	32	23	39	7
2[b]	none	56	23	22	38	4
	0.5 μg/ml	57	28	21	42	4
	1 mg/ml	56	24	20	41	5
	5 mg/ml	52	21	17	39	5
3	none	54	31	20	27	14
	0.5 μg/ml	63	42	24	19	15
	2.5 μg/ml	58	34	21	23	17
	1 mg/ml	61	34	21	23	16
	5 mg/ml	57	34	22	26	12

a. the number was shown as the percentage of positive cells.

b. PBMCs were not enough for all concentrations.

Effect of *C. nutans* on cytokine production

To investigate whether the extract was able to induce the release of cytokines. PBMCs were used to examine the inductive effect of *C. nutans* extract on IL-2 and IL-4 production. Using various concentrations of the extract, we found that the levels of IL-4 was increased in all samples at the final extract concentration of 2.5 and 5 mg/ml, whereas IL-2 levels were undetectable (< 16 pg/ml) at all concentrations of the extract tested (Table 4).

Table 4 Effect of *C. nutans* on the release of IL-2 and IL-4 from extract-treated peripheral blood mononuclear cells.

Donor #	Extract concentrations	IL-2[a]	IL-4[a]
4	none	< 16	< 16
	0.5 μg/ml	< 16	< 16
	5 μg/ml	< 16	< 16
	500 μg/ml	< 16	< 16
	1 mg/ml	< 16	< 16
	2.5 mg/ml	< 16	40
	5 mg/ml	< 16	1200
5	none	< 16	< 16
	0.5 μg/ml	< 16	< 16
	5 μg/ml	< 16	< 16
	500 μg/ml	< 16	< 16
	1 mg/ml	< 16	< 16
	2.5 mg/ml	< 16	< 16
	5 mg/ml	< 16	110
6	none	< 16	< 16
	0.5 μg/ml	< 16	< 16
	5 μg/ml	< 16	< 16
	500 μg/ml	< 16	< 16
	1 mg/ml	< 16	< 16
	2.5 mg/ml	< 16	66
	5 mg/ml	< 16	300

a. The levels were expressed in the units of pg/ml

DISCUSSION

We demonstrated the effect of *C. nutans* on lymphocyte proliferation, activity of NK cells, and cytokine production of human PBMCs. Enhancement of lymphocyte stimulation, quantified as stimulation index (S.I.), was observed in a dose-dependent response at the concentrations of 0.5 μg/ml, 2.5 μg/ml, and 5 μg/ml. The response was then gradually decreased at higher concentrations (Table 1). This may be due to the effect of extract or other factors induced in lymphocyte blastogenesis.

Considering the degree of lymphocyte reactivity, i.e., S.I., we found that there was no response with an S.I. greater than 2.0. This value is considered as positive mitogenic or antigenic responses (9). Therefore, the result suggested that *C. nutans* is likely to be a mitogen.

IL-4 has been reported to block the IL-2 - induced proliferation of peripheral blood cells and purified T cells (10). Its inhibition is confined to the naive T cell population. In our study we found the extract induced the release of IL-4 rather than IL-2 in the cultures especially at higher concentration of the extract. This may explain the lower degree of lymphocyte activation demonstrated in our system and a significant suppression of lymphocyte proliferation at the extract concentrations of 2.5 and 5 mg/ml.

We measured NK activity to examine the effect of the extract on one of natural defense mechanisms against a variety of infections. Our results showed a reduction in the function of NK cells. This may also be due to the effect of extract-induced IL-4 production since IL-4 has an inhibitory effect on cytoxicity of NK cells (10).

Additionally, we determined the survival of cells treated with various concentrations of *C. nutans* extract used in our studies, we found that the viability was greater than 95% at all concentrations (data not shown). Lower activities of the above parameters measured, therefore, were not resulted from the phenomenon of cell death or changes in lymphocyte subsets (Table 3).

We showed that there was no change in the numbers of lymphocyte subpopulations, i.e., $CD3^+$, $CD4^+$, $CD8^+$, $CD16^+$ / $CD56^+$ and $CD19^+$ cells in cultures containing *C. nutans* extracts as compared with the cultures without the extracts. Our finding was correlated with the *in vivo* study (6) which revealed no significant changes in $CD4^+$ and $CD8^+$ cells during the treatment. Other lymphocyte subsets were, however, not examined in their clinical trial study (6). According to lymphocyte subsets study, this suggested that *C. nutans* extract showed a selective effect on the function of lymphocyte subpopulations rather than regulating the number of each lymphocyte subset.

Cytokines are being increasingly recognized as essential mediators of normal and pathological immune responses (11). Although the number of study volunteers was small and the results were preliminary, these findings provided evidence that *C. nutans* may play an important role in the regulation of cytokines which are involved in the cascade of events that lead to a wide range of biological respones to exogenous and endogenous pathogens. Our study suggested the beneficial effecs of *C. nutans* in the treatment of HSV-2, i.e., shortening the duration of crust and healing stages, may be due to the role of IL-4 in anti-inflammatory activity (10).

Our results revealed evidence of alteration in non-specific cell-mediated immune responses resulting from the effect of *C. nutans*. Although this may lead to some possible explanations of how this traditional medicine has been useful for some viral infections (6). It is an *in vitro* study using normal human donors. Further studies should be performed to examine its effect on immunocompromised peripheral blood cells *in vitro* including *in vivo* study in either experimental animals or human volunteers.

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การศึกษาฤทธิ์ของสารสกัดจากพญายอต่อการตอบสนองทางภูมิคุ้มกันแบบเซลล์ในหลอดทดลอง

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บทคัดย่อ: สารสกัดพญายอมีฤทธิ์ในการรักษาอาการต่าง ๆ ที่เกิดจากการติดเชื้อและไม่ติดเชื้อ ได้ทำการศึกษาฤทธิ์ของสารสกัดพญายอต่อการตอบสนองทางภูมิคุ้มกันแบบเซลล์ในหลอดทดลองจากโมโนนิวเคลียร์เซลล์ (mononuclear cells) ของคน โดยทำการตรวจหาความสามารถของสารสกัดพญายอต่อการแบ่งตัวของลิมโฟซัยต์ (lymphocyte proliferation) การทำงานของเซลล์ที่ทำหน้าที่จับกินสิ่งแปลกปลอมแบบไม่จำเพาะ [natural killer (NK) cells] และการหลั่งของสาร interleukin-2 (IL-2) และ interleukin-4 (IL-4) ผลของการศึกษาพบว่าการแบ่งตัวของลิมโฟซัยท์เพิ่มขึ้นอย่างมีนัยสำคัญที่สารสกัดความเข้มข้น 0.5, 2.5 ไมโครกรัมต่อมิลลิลิตร แต่การแบ่งตัวนี้ลดลงอย่างมีนัยสำคัญเมื่อทำการเพาะเลี้ยงลิมโฟซัยท์โดยใช้สารสกัดที่ความเข้มข้น 2.5 และ 5 มิลลิกรัมต่อมิลลิลิตร นอกจากนี้การทำงานของเซลล์ NK นั้นพบว่าลดลงอย่างมีนัยสำคัญที่ความเข้มข้น 1 และ 5 มิลลิกรัมต่อมิลลิลิตร ในขณะที่ไม่พบการเปลี่ยนแปลงของปริมาณ IL-2 ที่หลั่งจากโมโนนิวเคลียร์เซลล์ในทุกความเข้มข้นของสารสกัดที่ใช้ทดสอบ แต่พบว่าปริมาณของ IL-4 เพิ่มขึ้นที่ความเข้มข้น 2.5 และ 5 มิลลิกรัมต่อมิลลิลิตร จากการศึกษานี้แสดงว่าสารสกัดพญายอซึ่งมีฤทธิ์เปลี่ยนแปลงการตอบสนองทางภูมิคุ้มกันแบบเซลล์ในหลอดทดลองนั้นอาจเป็นผลมาจากการเพิ่มขึ้นของ IL-4 ที่หลั่งมาจากโมโนนิวเคลียร์เซลล์

กุญแจคำ: พญายอ, ภูมิคุ้มกันแบบเซลล์