Effect of *Piper chaba* Hunter, *Piper sarmentosum* Roxb. and *Piper interruptum* Opiz. on Natural Killer Cell Activity and Lymphocyte Proliferation

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Background: *Immune system is the most important system of human body. Thai folk doctors have used some medicinal plants as an adaptogenic drug or immunomodulatory agent. Piper chaba Hunter, Piper sarmentosum Roxb. and Piper interruptum Opiz. are used by folk doctors to activate immune response in cancer patients.*
Objective: *To investigate the effect on natural killer cell activity and on lymphocyte proliferation activity of water extract of P. chaba Hunter, P. sarmentosum Roxb. and P. interruptum Opiz.*
Material and Method: *Plant materials were extracted by decoction method. All extracts were tested for an immunomodulatory effect using PBMCs from twelve healthy donors by chromium release assay. Lymphocyte proliferation was also determined by ^{3}H-thymidine uptake assay. The degree of activation was expressed as the stimulation index.*
Results: *The water extract of P. chaba Hunter significantly increased lymphocyte proliferation at concentrations of 1 ng/ml, 10 ng/ml, 1 μg/ml, 5 μg/ml, 10 μg/ml and 100 μg/ml. P. sarmentosum Roxb., and P. interruptum Opiz. extracts at those concentrations significantly stimulated lymphocyte proliferation. P. sarmentosum Roxb. extract significantly increased natural killer (NK) cell activity at a concentration of 100 μg/ml but P. chaba Hunter and P. interruptum Opiz. extracts did not significantly stimulate natural killer cell activity.*
Conclusion: *P. chaba Hunter, P. interruptum Opiz. and P. sarmentosum Roxb. have an immunomodulatory effect especially for P. sarmentosum Roxb. extract which can activate both lymphocyte proliferation and NK cell activity.*

Keywords: *Piper chaba Hunter, Piper interruptum Opiz., Piper sarmentosum Roxb., Immunomodulatory effect, NK cell, Lymphocyte proliferation*

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Immune system is the most important system in the body that protects against pathogens or foreign substances. It is separated into two distinct systems including innate and adaptive immune system. The components of innate immunity consist of physical barrier, complements, cytokines and cellular components such as natural killer cells, macrophages and dendritic cells. Adaptive immunity is a specific immune system and its cell components are T lymphocytes and B lymphocytes[1]. Immunomodulatory agents may act as a suppressor or modulator of immune system[2-4]. There are many plants which were inves-tigated immunodulatory activity by modulation the expression of cytokines such as tumor necrosis factor-α and interferon-γ, or by proliferation of peripheral blood mononuclear cells[5, 6].

Piper chaba Hunter, *Piper sarmentosum* Roxb. and *Piper interruptum* Opiz., are called Deeplee, Chaplu and Sakhan in Thai. All these plants are used in many Thai traditional remedies and used as daily food. They are composed in Benjakul preparation which was used as an adaptogenic drug for cancer patients[7]. *P. chaba* Hunter and *P. interruptum* Opiz. have been reported to have some pharmalogical activities such as an analgesic, anti-inflammatory, diuretic, anti-diarrheal and anti-pyretic activity[8,9]. The ethanolic

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extract of *P. chaba* Hunter, *P. sarmentosum* Roxb. and *P. interruptum* Opiz. have been found to be against human breast adenocarcinoma cell line (MCF-7) with IC_{50} of 35.17, 69.53 and 62.35 µg/ml, respectively[10]. The aqueous, methanol and hexane extract of *P. sarmentosum* Roxb. have strong antioxidant effect and could prevent cell apoptosis in H_2O_2-induced human umbilical vein endothelial cells[11]. Surprisingly, there is no report on natural killer (NK) cell activity and lymphocyte proliferation of three this plant extracts. Thus the aims of the present study were to investigate immunomodulatory effect of *P. chaba* Hunter, *P. sarmentosum* Roxb. and *P. interruptum* Opiz. extracts on NK cell activity and on lymphocyte proliferation activity.

Material and Method

Reagent

Penicillin-streptomycin solution and trypan blue were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). RPMI 1640 medium, fetal bovine serum (FBS), L-glutamine and 4-(2-hydroxyethyl)-1-piperazine than esulfonic/sulfuric acid (HEPES) were purchased from Invitrogen Life Technologies Inc. (Carlsbad, CA, USA). Ficoll-Hypaque mixture (Isoprep) was purchased from Robbins Scientific Corporation (Sunnyvale, CA, USA). ^{3}H-thymidine radio nucleotide and $Na_2\,^{51}CrO_4$ radio nucleotide were purchased from Perkin Elmer (Waltham, Massachusetts, USA). Dimethyl sulphoxide (DMSO) and other solvents were obtained from RCI Labscan Limited (Bangkok, Thailand).

Cell and cultures

A human erythroleukemic cell line, K562, (the American Type Culture Collection No. CCL-243) was cultured in complete media which composed by RPMI 1640 medium with 10 mM HEPES, 2 mM L-glutamine, 10% Fetal bovine serum, 100 U/ml of penicillin, and 100 µg/ml of streptomycin, and incubated at 37°C in an atmosphere containing 5% CO_2 for three days.

Preparation of peripheral blood mononuclear cells (PBMCs)

Twelve healthy donors were enrolled in this study. Each subject gave inform consent from of the present study. PBMCs were isolated from heparinized venous blood of healthy donors using Ficoll-Hypaque density gradient centrifugation and were suspended in RPMI 1640 medium with 10 mM HEPES, 2 mM L-glutamine, 10% fetal bovine serum, 100 U/ml of penicillin, and 100 µg/ml of streptomycin. The study was approved by the ethical review committees of faculty of medicine, Thammasat University under number 120/2555.

Preparation of crude extracts

The fruits of *Piper chaba* Hunter (collected from Amphor Kaosaming, Chantaburi), the roots of *Piper sarmentosum* Roxb. (collected from Amphor Jombueng, Ratchaburi) and the stems of *Piper interruptum* Opiz. (collected from Amphor Phoopan, Sakonnakhon) were collected by a Thai folk doctor on June 2010. They were identified by Southern Center of Thai Medicinal plant at Faculty of Pharmaceutical Science, Prince of Songkla University, Songkhla, Thailand. Some plant materials were deposited at the herbarium of Prince of Songkla University which were shown the herbarium voucher specimen number and traditional used in Table 1. These plants were cleaned, cut into small pieces, dried in oven at 50°C and grinded to powder. The water extract was obtained by boiling plant material with distilled water for 30 min, filtered and then dehydrated by using lyophilizer. The percentage yield of water extract was calculated and show in Table 1.

Natural killer cell (NK) activity assay[12]

In brief, PBMCs were washed twice with 10 ml complete media and adjusted to $2x10^6$ cells/ml in complete media. PBMCs suspensions (500 µl) were placed into each polystyrene tube. Complete media (500 µl) containing different concentrations of the water extracts were added. The final concentrations of extracts were: 10 ng/ml, 100 ng/ml, 1 µg/ml, 10 µg/ml and 100 µg/ml, respectively. The cells were incubated in the presence or absence of samples or media control at 37°C in an incubator under an atmosphere containing 5% CO_2 for 18-24 hours. After incubation, the cultures were washed with 1 ml complete media and then used as effector cells. K562 cells labeled with 100 µCi of $Na_2\,^{51}CrO_4$ were used as target cells. These labeled target cells 100 µl ($2x10^4$ cells/ml) were incubated with effector cells, in triplicate for each concentration, for 4 hours at 37°C in an incubator under an atmosphere containing 5% CO_2. Effector cells to target cell ratio used were: 90:1, 30:1, 10:1 and 3:1. After incubation, 100 µl/well of supernatants from each well were transferred into microtiter tubes and counted in a multigamma counter (Automatic Gamma Counter, Perkin Elmer, Massachusetts, USA).

The percentage of cytolysis was calculated according to the formula:

$$\% \text{ cytolysis} = \frac{(\text{experimental release - spontaneous release})}{(\text{maximal release - spontaneous release})} \times 100$$

Spontaneous release was measured by incubation of target cells with media alone, while maximal release was measured by incubation of target cells with 5% triton x-100. NK cells activity was expressed as a lytic unit (LU)/10^7 PBMCs. One LU was defined as the number of effector cells required for 20% specific lysis of $1x10^4$ target cells.

***Lymphocyte proliferation assay*[12]**

Briefly, 100 µl PBMCs ($2x10^6$ cells/ml) were cultured in 96 well microtiter plates with plant extracts. The final concentrations of these samples were 1 ng/ml, 10 ng/ml, 100 ng/ml, 1 µg/ml, 5 µg/ml, 10 µg/ml and 100 µg/ml. Incubation was at 37°C with 5% CO_2 for 54 hours. After incubation, 20 µl ^{3}H-thymidine (25 mCi/ml) was added into each 96 well plates and incubated at 37°C with 5% CO_2 for 18 hours. Lymphocyte proliferation was determined by ^{3}H-thymidine uptake, which was measured by a liquid scintillation counting using Topcount Microplate Scintillation & Luminescence Counter (Packard Instrumental Co, CT, USA). The degree of activation was expressed as the stimulation index.

The stimulating index was calculated according to the formula:

$$\text{Stimulating index} = \frac{^3\text{H-thymidine uptake of sample with extract}}{^3\text{H-thymidine uptake of sample without extract}}$$

Statistical analysis

For statistical analysis, the values are expressed as mean ± SEM. The statistical significant was calculated by Student's paired t-test and statistical significant was defined as $p \leq 0.05$.

Results

The effect of water extracts of *P. chaba* Hunter (PCW), *P. sarmentosum* Roxb. (PSW) and *P. interruptum* Opiz. (PIW) on the proliferation of human lymphocytes are shown in Fig. 1. The water extract of *P. chaba* Hunter significantly increased lymphocyte proliferation at concentrations of 1 ng/ml, 10 ng/ml, 1 µg/ml, 5 µg/ml, 10 µg/ml and 100 µg/ml, whereas no significant lymphocyte proliferation enhancement was found at concentration of 100 ng/ml. *P. sarmentosum* Roxb. and *P. interruptum* Opiz. extracts at all concentrations (10 ng/ml-100 µg/ml) significantly stimulated lymphocyte proliferation.

For the effect of water extracts of *P. chaba*

Table 1. The summarize data of *P. chaba* Hunter, *P. sarmentosum* Roxb. and *P. interruptum* Opiz.

Plants	Thai name	Part of used	% yield of extracts	Voucher number	Thai traditional used[24]	Biological activity
P. chaba Hunter	Deeplee	Fruit	17.9	SKP 146160301	Treatment of carminative	Anticancer[10,25] Anti-inflammatory[8,9] Antimalarial[26] Immunomodulatory activity[18] Analgesic[8,9] Anti-diarrhoeal[8] Antipyretic[9] Anti-amoebic[27]
P. sarmentosum Roxb.	Chaplu	Root	15.54	SKP 146161901	Treatment of carminative, digestive, expectorants	Anti-cancer[10] Anti-amoebic[27] Anti-oxidation[11]
P. interruptum Opiz.	Sakhan	Stem	11.93	SKP 146160901	Treatment of carminative	Anticancer[10] Anti-inflammatory[9] Analgesic[9] Antipyretic[9]

Hunter (PCW), *P. sarmentosum* Roxb. (PSW) and *P. interruptum* Opiz. (PIW) extracts on natural killer cell activity, *P. sarmentosum* Roxb. extract significantly increased the natural killer cell activity at 100 µg/ml concentration but it did not show any significant stimulating effect on NK cell activity at lower concentrations (10 ng/ml-10 µg/ml). *P. chaba* Hunter and *P. interruptum* Opiz. extracts had no significantly stimulating effect on NK cell activity (Fig. 2).

Discussion

Many plant extracts were evaluated for the potent immunomodulatory effect on lymphocyte proliferation and the development to be an immunomodulatory agent[13-15]. Lymphocytes are important cells in the immune system that include NK cells, T cells and B cells. Thus, the role of lymphocytes is detected when the body obtains microorganisms or foreign substances. Lymphocyte proliferation will be then stimulated and they react against infectious microorganisms and foreign substances[16]. In the present study, we examined immunomodulatory activity of water extract of *P. chaba* Hunter, *P. sarmentosum* Roxb. and *P. interruptum* Opiz. as adaptogenic drug for cancer treatment[17] was found that all these plant extracts activated lymphocyte proliferation but only *P. sarmentosum* Roxb. extract was also increased NK cell activity. This result is similar to a previous report, which tested in plant in family Piperaceae. Oral administration of methanolic extract of *P. longum* Linn. in a dose of 10 mg w/w or its pure compound as piperine, at a concentration of 1.14 mg w/w was found to increase the total white blood cells count in mice with 142.8 and 138.9% respectively, when compare before treatment[18].

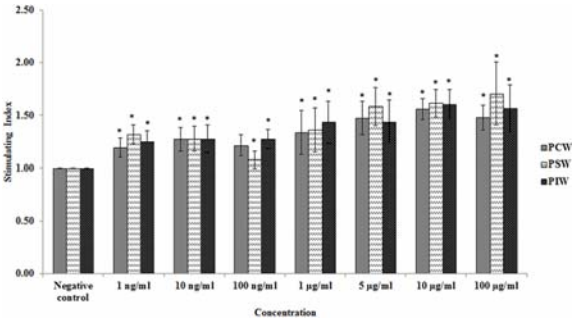

Fig. 1 Effect of *P. chaba* Hunter (PCW), *P. sarmentosum* Roxb. (PSW) and *P. interruptum* Opiz. (PIW) on lymphocyte proliferation. Significant different between negative control group and experimental group, **p*-value ≤0.05 (n = 12, Mean ± SEM).

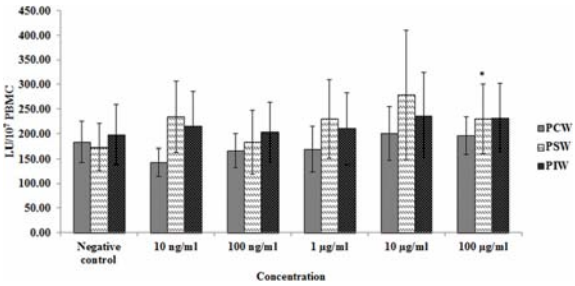

Fig. 2 Effect of *P. chaba* Hunter (PCW), *P. sarmentosum* Roxb. (PSW) and *P. interruptum* Opiz. (PIW) on NK activity. Significant different between negative control group and experimental group, **p*-value ≤0.05 (n = 12, Mean ± SEM).

On the other hand, piperine can be significantly reduced T cells in mice at high concentration of 4.5 mg/kg[19]. Piperine is a main organic compound which is isolated from alcoholic extract of plants in the family Piperaceae[20,21]. However, all plant extracts used in the present results were extracted by the decoction method. The significantly stimulation of lymphocyte proliferation activity found in these plant extracts may result from the compound containing in the extracts which can increased the secretion of cytokines (such as IL-2) which are necessary for the differentiation and proliferation of lymphocyte[22]. The water extract of these plants showed proliferation as a dose response manner and *P. sarmentosum* Roxb. showed the highest stimulation index. For NK cells activity assay, only an aqueous extract of *P. sarmentosum* Roxb. significantly stimulated NK cells activity which had not previously been reported in all these plants. As an aqueous extract of *P. sarmentosum* Roxb. has the highest immuno-modulatory effect on both activity on lymphocyte proliferation and NK cell activity which plays an important role to destroy tumor cells[23], an aqueous extract of *P. sarmentosum* Roxb. roots should be promoted as immunomodulatory agent on NK cell activity. In addition, all of these extract are also promoted as stimulating lymphocyte proliferation agents. These obtained results are new knowledge for using these three species of *Piper* besides their used as ingredients in food especially for an aqueous extract of *P. sarmentosum* Roxb. roots. Normally, *P. sarmentosum* Roxb. leaves is used as vegetable whereas its root was used as Thai traditional drug. Thus, the leaves of *P. sarmentosum* Roxb. should be continuously studied on their imunomodulatory effect and compared with that of the root of *P. sarmentosum*

Roxb. Although the water extract of these three plants will be useful to promote as a vegetable, it should be also promoted as an immune enhancing health food for cancer patients. These results support the use of these plants as an adaptogenic drug for cancer patients by Thai traditional doctors.

Conclusion

In conclusion, the water extracts of *P. chaba* Hunter, *P. interruptum* Opiz. and *P. sarmentosum* Roxb. have an immunomodulatory effect especially for *P. sarmentosum* root extract, which can also activate lymphocyte proliferation and NK cells activity. Thus, the water extract of *P. sarmentosum* Roxb., *P. chaba* Hunter and *P. interruptum* Opiz. may contain compounds which have effect on the immune system. These results support in using these plants as an immunomodulatory agent. However, in vivo studies of these plants for immunomodulatory activity have to be studied further.

What is already known on this topic?

P. chaba Hunter, *P. interruptum* Opiz. and *P. sarmentosum* Roxb. were composed in Thai medicinal preparation for treatment in cancer patients by Thai folk doctors. Their effect may stimulate the immune system involving tumor cells. *P. chaba* Hunter were reported on about its lymphocyte proliferation activity in animal models[18], but there is no report about its NK cell activity. For *P. interruptum* Opiz. and *P. sarmentosum* Roxb. there are still no previous scientific reports about lymphocyte proliferation and NK cell activity.

What this study adds?

The present study found that aqueous extract of *P. Chaba* Hunter, *P. interruptum* Opiz. and *P. sarmentosum* Roxb. significantly stimulated lymphocyte proliferation. Moreover *P. sarmentosum* Roxb. can also activate NK cell activity. This result can support using all these plants as an adaptogenic drug for cancer patients.

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Potential conflicts of interest

None.

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ผลของ Piper chaba Hunter, Piper sarmentosum Roxb. และ Piper interruptum Opiz. ต่อการกระตุ้นการทำงานของเนเซอรัลคิลเลอร์เซลล์และการแบ่งตัวของเม็ดเลือดขาวลิมโฟไซต์

สุมาลี ปานทอง, อรุณพร อิฐรัตน์

ภูมิหลัง: *ระบบภูมิคุ้มกันเป็นระบบที่สำคัญของร่างกายมนุษย์ หมอพื้นบ้านของไทยมีการใช้สมุนไพรเป็นยาปรับธาตุหรือปรับระบบภูมิคุ้มกัน Piper chaba Hunter, Piper sarmentosum Roxb. และ Piper interruptum Opiz. ถูกนำใช้เป็นส่วนประกอบในตำรับยาปรับธาตุสำหรับผู้ป่วยมะเร็ง ซึ่งน่าจะมีฤทธิ์กระตุ้นการตอบสนองของภูมิคุ้มกัน*

วัตถุประสงค์: *เพื่อศึกษาผลของสารสกัดด้วยน้ำของ Piper chaba Hunter, Piper sarmentosum Roxb. และ Piper interruptum Opiz. ต่อการทำงานของเนเซอรัลคิลเลอร์เซลล์และการแบ่งตัวของเม็ดเลือดขาวลิมโฟไซต์*

วัสดุและวิธีการ: *ตัวอย่างพืชถูกนำไปสกัดด้วยวิธีการต้มน้ำสารสกัดทั้งหมดถูกนำมาทดสอบฤทธิ์กระตุ้นภูมิคุ้มกันโดยใช้จากอาสาสมัครสุขภาพดี 12 ราย ด้วยเทคนิคการปลดปล่อยสารรังสีโครเมี่ยมและเทคนิคการนำสารรังสีไทมิดีนเข้าสู่เซลล์*

ผลการศึกษา: *สารสกัดชั้นน้ำของ P. chaba Hunter กระตุ้นการเพิ่มจำนวนของเม็ดเลือดขาวลิมโฟไซต์ได้อย่างมีนัยสำคัญที่ความเข้มข้น 1 นาโนกรัม/มิลลิลิตร, 10 นาโนกรัม/มิลลิลิตร, 1 ไมโครกรัม/มิลลิลิตร, 5 ไมโครกรัม/มิลลิลิตร, 10 ไมโครกรัม/มิลลิลิตร และ 100 ไมโครกรัม/มิลลิลิตร สารสกัด P. sarmentosum Roxb. และ P. interruptum Opiz. ที่ทุกความเข้มข้นกระตุ้นการเพิ่มจำนวนของเม็ดเลือดขาวลิมโฟไซต์อย่างมีนัยสำคัญทางสถิติ สารสกัด P. sarmentosum Roxb. ยังมีฤทธิ์กระตุ้นการทำงานของเนเซอรัลคิลเลอร์เซลล์ที่ความเข้มข้น 100 ไมโครกรัม/มิลลิลิตร อย่างมีนัยสำคัญทางสถิติแต่สารสกัด P. chaba Hunter และ P. interruptum Opiz. ไม่มีผลกระตุ้นการทำงานของเนเซอรัลคิลเลอร์เซลล์ได้*

สรุป: *P. chaba Hunter, P. interruptum Opiz. และ P. sarmentosum Roxb. มีผลกระตุ้นระบบภูมิคุ้มกันโดยเฉพาะ P. sarmentosum Roxb. ที่สามารถกระตุ้นทั้งการแบ่งตัวของเม็ดเลือดขาวลิมโฟไซต์และการทำงานของเนเซอรัลคิลเลอร์เซลล์ได้*