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In vitro immunomodulatory activities of extracts from some Thai medicinal plants

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Abstract

Eleven medicinal plants commonly used in Thai tradition were extracted with either chloroform, methanol or water. Thirty six crude extracts were screened for activating human lymphocytes and phagocytes. Using flow cytometry, the marker used for detecting lymphocyte activation and proliferation were CD69 antigen expression. Effects on phagocytes were detected by measuring the migration of polymorphonuclear cells towards a chemoattractant under agarose. Four chloroform extracts from *Alpinia galanga*, *Boesenbergia pandurata*, *Piper chaba* and *Zingiber zerumbet* stimulated T lymphocytes to express CD69 antigen (19.6%, 21.0%, 29.0% and 31.6% respectively) compared to the control (6.2%). *Boesenbergia pandurata* extract was further purified and 1 of 3 fractions induced CD69 expression at the concentration of 50 and 100 μg/ml. None of the crude extracts stimulated the migration of polymorphonuclear cells but one chloroform extract from *Zingiber zerumbet* and 3 methanol extracts from *Eclipta prostrata*, *Piper betle* and *Zingiber zerumbet* suppressed polymorphonuclear cells migration.

Keywords: Immunomodulatory activity; Medicinal plant; Lymphocyte activation; Polymorphonuclear cells migration

1. Introduction

In Thailand, since ancient time, many kinds of medicinal plants have been used in folk medicine to combat diseases directly or to relieve some of their symptoms. This knowledge is handed down from one generation to the next and their continued use is attractive because they have no side effects and they are cheap and easy to obtain. In addition they must be effective at relieving some symptoms otherwise their use would have been discontinued long ago. Many of them have been reported to have antimicrobial activity such as those from *Alpinia galanga, Barleria lupulina, Boesenbergia pandurata, Eclipta prostrata, Murraya paniculata, Piper betle, Piper chaba, Spilanthes acmella* and *Zingiber zerumbet,* (Fransworth and Bunyapraphatsara, 1992; Mondol et al., 2001; Rajan et al., 2002; Rukachaisirikul et al., 2002; Bendjeddou et al., 2003; Wiart et al., 2004). Others have been reported to have anti-inflammatory activity such as those from *Acanthus ebracteatus*, *Barleria lupulina, Boesenbergia pandurata*, *Coccinia grandis* and *Spilanthes acmella*, (Jawaweera, 1981; Fransworth and Bunyapraphatsara, 1992; Kanchanapoom et al., 2001; Rajan et al., 2002) and still others have anticancer activity such as those from *Alpinia galanga, Zingiber zerumbet* (Itokawa et al., 1987; Huang et al., 2005). In general, their modes of action have never been scientifically identified or even investigated. In some cases their activities may be mediated by interaction with the host's immune system and facilitate the elimination of potential pathogens or involve in host protective mechanisms which have been shown in many kinds of various extracts from medicinal plants (Van der Nat et al., 1987; Nores et al., 1997; Wagner, 1990; Benencia et al., 2000; Ko et al., 2004).

Phagocytosis and lymphocyte activation are two important processes involved in immunity and protection against diseases. Phagocytosis is one of the first line defense mechanisms against invasion by a pathogen. It is mediated by polymorphonuclear cells (PMNs) and macrophages. Phagocytic cell movement towards an infective agent resulting from chemotaxis is the first stage of phagocytosis. Therefore, increasing the chemotactic activity can enhance the ability of cells to eliminate potential pathogens. Lymphocytes play an important role in specific immunity by recognizing specific antigens that are foreign to the host. Lymphocyte activation can be evaluated by determination of CD69 expression on the cell surface. The CD69 antigen is one of the earliest markers expressed by activated lymphocytes following stimulation by a variety of agents such as pharmacological agents, plant lectins and microbial proteins (Cebrian et al., 1988; Hara et al., 1986; Testi et al., 1989; Maino et al., 1995). In this work, extracts of 11 different plants commonly used in Thai folk medicine were prepared and tested for their effects on chemotactic activity and CD69 expression on lymphocytes.

2. Materials and methods

2.1. Plant material

Eleven different plants were collected in an area of Songkhla Province, southern Thailand (Table 1). The voucher specimens have been deposited at the Department of Pharmacognosy and Pharmaceutical Botany, Prince of Songkla University, Thailand. The preparation of plants and extraction procedures has been described by Tewtrakul et al (2003). Briefly, dried plants were successively extracted with either chloroform, methanol and hot water and solvents were removed under reduced pressure. Thirty six crude extracts were obtained for this assay. Stocks of all extracts were made by dissolving them in either chloroform or dimethyl sulfoxide (DMSO) and steriled by passing through 0.45 μm syringe filter. For testing, the stocks were diluted to a final concentration of 100 and 1,000 μg/ml with

culture medium. Diluents themselves were shown to have no effects on the test systems used. Extract that exhibited immunomodulatory activity was fractionated on a column of silica gel with mixtures of n-hexane, chloroform and methanol by increasing polarity of eluting solvents (Unger,1990). Each fraction was collected, evaporated under reduced pressure and spotted on thin layer chromatography. Fractions that gave similar patterns were pooled together and further investigated.

2.2. Polymorphonucleas cells preparation

Human whole blood was collected in sodium heparinized tubes. Polymorphonuclear cells (PMNs) were isolated by Ficoll-Histopaque (Sigma, USA) density gradient centrifugation (Ferrante and Thong, 1989). Before assay, cells were washed twice with Dulbecco PBS + 1% glucose and adjusted to $4x10^7$ cells/ml in medium-199 containing 10% fetal calf serum.

2.3. Polymorphonuclear cells migration

The assay for the migration of PMNs in agarose was performed as previously described (Vuddhakul et al., 1989). Briefly, 3 ml of double strength medium-199 containing 10% heat-inactivated fetal calf serum was mixed with 3 ml of 2% agarose solution and poured into 60x15 mm tissue culture plates. Wells of 2 mm diameter and 2.5 mm apart were punched in sets of three. The middle well received 5 µl of a PMNs suspension at a concentration of $4x10^7$ cells/ml, and the test extract at a final concentration of 100 µg/ml. The outer well received a chemoattractant, and the inner well received medium-199 only. All tests were done in triplicate. The chemoattractant was made by incubating $1x10^7$ *Candida albicans* cells with 2 ml fresh human serum for 30 min at 37°C. This was centrifuged at 1,000 *g* for 10 min, and the activated serum removed for use.

The agarose plates were kept in an air atmosphere with 5%CO_2 and high humidity for 2h, and the distance phagocytes moved towards the inner well (random movement) and the outer well (chemotaxis) was measured with an inverted microscope and ocular micrometer. Results are expressed as mean units/2h $\pm$ SD.

2.4. Stimulation of Lymphocytes

Human whole blood was collected with added sodium heparin anticoagulant. Various lymphocyte stimuli, phytohemagglutinin (PHA), staphylococcal enterotoxin B, (SEB) and phorbol 12-myristate acetate (PMA) (Maino et al., 1995; Suzuki et al., 1999; Klein et al., 1997) were used to determine the best achievable lymphocyte activation. Briefly, PHA (Sigma) a plant lectin, which is a non-specific stimulus was incubated with whole blood at a final concentrations of 5, 10 and 25 µg/ml. SEB (Sigma), a superantigen, together with the co-stimulatory, anti CD28, was added to whole blood at a final concentrations of 5, 10 and 25 µg/ml. PMA, (Sigma), pharmacologic agent, in a combination with ionomycin (Sigma) (Baran et al., 2001) was cultured with whole blood at final concentrations of 5, 10 and 25 ng/ml. The culture tubes were incubated in a humidified, 37°C, 5% CO_2 incubator for 4 h and CD69 expression was determined. The best lymphocyte stimulus was selected and further investigated for the optimum time of activation at either 4, 8, 12 or 24 h.

2.5. Screening of plant extracts for lymphocyte activation

Human whole blood was incubated with test extract at a final concentration of 1,000 µg /ml or with test fractions at 10, 50 and 100 µg/ml at 37°C in a 5%CO_2 incubator. The expression of CD69 after lymphocyte stimulation was determined by two-color immunofluorescent staining (Maino et al., 1995). Briefly, 50 µl of whole blood was lysed in 0.5 ml of FACS™ Lysing Solution (Becton Dickinson) for 10 min at room temperature. Cells were washed in PBS and stained with fluorescent conjugated antibodies consisting of CD4 per CP and CD69 PE for 30 min at room temperature in the dark. After staining, samples were washed and fixed in 1% paraformaldehyde in PBS and stored at 4°C until FACS analysis. Flow cytometric analysis was performed on a FACS Calibur™ flow cytometer (Becton Dickinson). Data were acquired using CELL-Quest™ software (Becton Dickinson). Typically, collecting 10,000 gated lymphocytes using forward scatter and side scatter and using CD4 + event as a fluorescent trigger for CD69 expression. Data were displayed as two-color dot plots to measure the proportion of the double-positive (CD4 +/ CD69 +) cells.

2.6. Statistic analysis

The student's *t* test was used to compare the different between tested and control results

3. Results

3.1. Stimulation of Lymphocytes

Various concentrations of PHA, SEB and PMA were evaluated for stimulating CD69 expression on lymphocytes. It was found that PHA gave the best stimulus (Table 2) and there was no significant differences in lymphocyte stimulation with PHA concentrations of 5, 10 and 25 µg/ml. PHA at 5 µg/ml was then used to determine the optimum time for activation. As illustrated in Fig.1, there was no significant difference in CD69 expression after either 4, 8, 12 or 24 h incubation. All further experiments were therefore performed with a 4h incubation period.

3.2. Effect of crude extracts on CD69 expression

Thirty six crude extracts from 11 medicinal plants, each at a final concentration of 1,000 µg/ml were tested for lymphocyte stimulation as measured by CD69 expression. For recognition of a significant stimulation results were compared to the stimulation obtained with PHA. Extracts from 4 medicinal plants, *Alpinia galanga, Boesenbergia pandurata, Piper chaba* and *Zingiber zerumbet*, produced significant stimulation of lymphocytes (Table 3). CD69 expression on lymphocytes was detected only with chloroform extracts of *Alpinia galanga, Boesenbergia pandurata, Piper chaba* and *Zingiber zerumbet* with values of 19.6%, 21.0%, 29.0% and 31.6 % respectively. Non-stimulated control had an increase of 6.2% and PHA gave a 45.5% stimulation. *Boesenbergia pandurata* was fractionated and fractions were tested for stimulation of CD69 expression. Three fractions of *Boesenbergia pandurata* extract showed some lymphocyte activation but only 1 fraction gave significant stimulation at concentration of 50 and 100 µg/ml in a dose response manner (Fig.2).

3.3. Effect of crude extracts on PMNs migration

All 36 crude extracts from the 11 medicinal plants at a final concentration of 100 μg/ml were tested for their effect on the migration rate of PMNs. No stimulation of the migration rate was detected. However, 4 extracts that included the chloroform extract of *Zingiber zerumbet*, 3 methanol extracts of *Eclipta prostrata, Piper betle* and *Zingiber zerumbet* showed suppression of PMN migration. (Fig.3). In addition, 3 extracts, a chloroform extract from *Murraya maniculata* and *Piper betle* and a methanol extract from *Alpinia galanga* were toxic and caused cells lysis.

4. Discussion

In this study, chloroform extracts from 4 medicinal plants, *Alpinia galanga, Boesenbergia pandurata, Piper chaba* and *Zingiber zerumbet* induced activation of human lymphocytes. This is the first report of such an effect. This activation could be related to some of the benefits that seem to result from taking these folk medicines. It would be therefore of interest to see if some of their uses in traditional medicine could be related to these lymphocyte activations. *Alpinia galanga* has been used to treat problems associated with the digestive system, relieving bronchitis, flatulence, and acts against fungi and itching (Brown, 1995). The pungent principal compound of *Alpinia galanga*, 1'-acetoxychavicol acetate, has been reported to possess antitumor, anti-inflammatory, antifungal, gastroprotective, and xanthine oxidase inhibitory activities (Itokawa et al., 1987; Noro et al., 1988; Nakamura et al.,1998; Yang et al., 1999; Matsuda et al., 2003b). Aqueous and acetone extracts of the rhizomes of *Alpinia galanga* were found to exhibit antiallergic properties. (Matsuda et al., 2003a). In addition, hot water extracts of *Alpinia galanga* containing some polysaccharide, increased the number of peritoneal exudate cells and spleen cells of mice. It also markedly enhanced the proliferation of murine spleen cells (Bendjeddou et al., 2003). In this study, only a chloroform extract of *Alpinia galanga* rhizomes had immunostimulating ability on human lymphocytes. We could not detect any activity of its aqueous extract. This difference may be due to the different methods of extraction used as their polysaccharide water extract had been partially purified by precipitation twice with ethanol and all protein had been removed by trichloroacetic acid and sodium acetate. It is also of interest that *Alpinia galanga* is widely used in India, China and Southeast Asian countries, such as Thailand, Indonesia, and Philippines as a spice or ginger substitute for flavoring foods.

Boesenbergia pandurata, currently known as *Boesenbergia rotunda* (Larsen,1996), a perennial herb of the family Zingiberaceae. The fresh rhizome is also used in cooking, and in Thai folk medicine as an aphrodisiac, and for the treatment of colic disorders. In this study, a crude chloroform extract of yellow rhizomes enhanced lymphocyte activity (Table 3). The activity was concentrated in 1 of 3 fractions obtained during purification (Fig. 2).

P. chaba is a climbing, glabrous creeper, cultivated in various parts of India and Malaya Island (Kirtikar et al., 1935). The roots and fruits find numerous applications in medicine. They are particularly useful in treating asthma, bronchitis, fever, and in the abdomen, as a stimulant and in the treatment of haemorrhoids (Kirtikar et al., 1935). In this study, we found that only a chloroform crude extract of the fruit of *Piper chaba* exhibited lymphocyte activation.

A crude chloroform extract of the rhizome of *Zingiber zerumbet*, also activated lymphocytes. This rhizome plant is used in traditional medicine in some Southeast Asian countries, as an anti-inflammatory agent (Murakami et al., 1999). There have been few reports on this plant and its constituents. However, one of its compounds is claimed to prevent colon and skin cancer (Tanaka et al., 2001; Murakami et al., 2003; Murakami et al., 2004).

In this study, none of the 36 crude extracts activated polymorphonuclear cells migration but 4 extracts suppressed migration and 3 extracts caused cell lysis (Fig. 3). Hence, none of these plant extracts are likely to assist in the first line of protection against any microbial

infection and some may harm the immune cells that might normally combat infection. However, their suppression activity may act as an anti-inflammatory agent in some diseases.

In conclusion, we have established that chloroform extracts from 4 of 11 medicinal plants used in Thai traditional medicine possessed lymphocyte stimulating activity. In view of the many cases of human sicknesses where these plants have been used for treatment it would not be surprising if many of the beneficial effects were derived from the activation of the hosts immune systems. This confirms that the use of these medicinal plants will continue to have benefits for the treatment of various sicknesses perhaps especially those associated with the elderly or cancer and AIDS patients.

Among these 4 medicinal plants that enhance immunity, the extracts from *Boesenbergia pandurata* gave the most promising result as it is popular in health promotion and aphrodisiac (Fransworth and Bunyapraphatsara,1992) and in this study they had no effect on PMNs whereas the chloroform extract of *Alpinia galanga* caused PMNs to lyse (Fig. 3) and chloroform and methanol extracts of *Zingiber zerumbet* suppressed PMNs migration. Purification by fractionation confirmed its activity in a dose response manner (Fig. 2). Further re-fractionation and structural determination of the active components of *Boesenbergia pandurata* including in vivo investigations, are progressing in order to confirm their immunostimulatory activities and hopefully to help elucidate their mechanisms of action.

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References

Baran, J., Kowalczyk, D., Ôzog, M., Zembala, M., 2001. Three-color flow cytometry detection of intracellular cytokines in peripheral blood mononuclear cells: Comparative analysis of phorbol myristate acetate-ionomycin and phytohemagglutinin stimulation. Clinical and Diagnostic Laboratory Immunology 8, 303-313.

Bendjeddou, D., Lalaoui, K., Satta, D., 2003. Immunostimulating activity of the hot water-soluble polysaccharide extracts of *Anacyclus pyrethrum*, *Alpinia galanga* and *Citrullus colocynthis*. Journal of Ethnopharmacology 88, 155-160.

Bernencia, F., Courreges, M.C., Coulombie, F.C., 2000. In vivo and in vitro immunomodulatory activities of *Trichilia glabra* aqueous leaf extracts. Journal of Ethnopharmacology 69, 199-205

Brown, D., 1995. In: Kinderstey, D. (Ed.), The Royal Horticultural Society Encyclopedia of Herbs and Their Uses. London, pp. 235–237.

Cebrian, M., Yague, E., Rincon, M., Lopez-Botet, M., de Landazuri, M.O., Sanchez-Madrid, F., 1988. Triggering of T cell proliferation through AIM, an activation inducer molecule expressed on activated human lymphocytes. Journal of Experimental Medicine 168, 1621-1637.

Fransworth, N.R., Bunyapraphatsara, N., 1992. Thai medicinal plants recommended for primary health care system. Prachachon, Bangkok, pp. 44, 70,.72, 193, 229, 261.

Ferrante, A., Thong, Y.H., 1980. Optimal conditions for the simultaneous purification of mononuclear and polymorphonuclear leukocytes from human blood. Journal of Immunology 36, 108-117.

Hara, T., Jung, L.K., Bjorndahl, J.M., Fu, S.M., 1986. T cell activation III. Rapid induction of a phosphorelated 28kD/32kD disulfide-linked early activation antigen (EA1) by 12-*O*-tetradecanoyl phorbal-13-acetate mitogens and antigens. Journal of Experimental Medicine 164, 1988-2005.

Huang, G.C., Chien, T.Y., Chen, L.G., Wang, C.C., 2005. Antitumor effects of zerumbone from *Zingiber zermbet* in P-388D1 cells in vitro and in vivo. Planta Medica 71, 219-224.

Itokawa, H., Morita, H., Sumitomo, T., Totsuka, N., Takeya, K., 1987. Antitumour principles from *Alpinia galanga*. Planta Medica 53, 32-33.

Jayaweera, D.M.A., 1981. Medicinal Plants Used in Ceylon. Part III. A publication of National Science Council of Sri Lanka, Colombo, Sri Lanka, pp.71.

Kanchanapoom, T., Kasai, R., Picheansoonthon, C., Yamasaki, K., 2001. Megastigmane, aliphatic alcohol and benzoxazinoid glycosides from *Acanthus ebracteatus*. Phytochemistry 58, 811-817.

Kirtikar, K.R., Basu, B.D., 1935. Indian Medicinal Plants. Vol III. 2nd ed. Lalit Mohan Basu Publications, Allahabad, India, pp. 2130.

Klein, S.A., Dobmeyer, J.M., Dobmeyer, T.S., Pape, M., Ottmann, O.G., Helm, E.B., Hoelzer, D., Rossol, R., 1997. Demonstration of the Th1 to Th2 cytokine shift during the course of HIV-1 infection using cytoplasmic cytokines detection on single cell level by flow cytometry. AIDS 11, 1111-1118.

Ko, E., Rho, S., Lee, E., Seo, Y., Cho, C., Lee, Y., Min, B., Shin, M., Hong, M., Bae, H., 2004. Traditional Korean medicin (SCRT) modelate Th1/Th2 specific cytokines production in mice CD4+ T cell. Journal of Ethnopharmacology 92, 121-128.

Larsen, K., 1996. A preliminary checklist of the Zingiberaceae of Thailand.Thai Forest Bulletin (Botany) 24, 35–49.

Maino, V.C., Suni, M, A., Ruitenberg, J.J., 1995. Rapid flow cytometric method for measuring lymphocyte subset activation. Cytometry 20, 127-133.

Matsuda, H., Morikawa,T., Managi, H., Yoshikawa, M., 2003a. Antiallergic principles from *Alpinia galanga*: structural requirements of phenylpropanoids for inhibition of degranulation and release of TNF- alpha and IL-4 in RBL-2H3 cell. Bioorganic and Medical Chemistry Letters 13, 3197-3202.

Matsuda, H., Pongpiriyadacha, Y., Morikawa, T., Ochi, M., Yoshikawa, M., 2003b. Gastroprotective effects of phenylpropanoids from the rhizomes of *Alpinia galanga* in rats: structural requirements and mode of action. European Journal of Pharmacology 471, 59-67.

Mondal, S.K., Ray, B., Ghosal, P.K., Telaman, A., Vourinen, T., 2001. Structural features of a water soluble gum polysaccharide from *Murraya paniculata* fruites. International Journal of Biological Macromolecules 29, 169-174.

Murakami, A., Takahashi, M., Jiwajinda, S., Koshimizu, K., Ohigashi, H., 1999. Identification of zerumbone in *Zingiber zerumbet* Smith as a potent inhibitor of 12-*O*-tetradecanoylphorbol-13-acetate-induced Epstein-Barr virus activation. Bioscience Biotechnology Biochemistry 63, 1811–1812.

Murakami, A., Hayashi, R., Tanaka., T., Kwon, K.H., Ohigashi, H., Safitri, R., 2003. Suppression of dextran sodium sulfate-induced colitis in mice by zerumbone, a subtropical ginger sesquiterpene, and nimesulide: separately and in combination. Biochemical Pharmacology 66, 1253-1261.

Murakami, A., Tanaka, T., Lee, J.Y., Surh, Y.J., Kim, H. W., Kawabata, K., Nakamura, Y., Jiwajinda, S., and Ohigashi, H., 2004. Zerumbone, a sesquiterpene in subtropical ginger, suppresses skin tumor initiation and promotion stages in ICR mice. International Journal of Cancer 110, 481–490.

Nakamura, Y., Murakami, A., Ohto, Y., Torikai, K., Tanaka, T., Ohigashi, H., 1998. Suppression of tumor promoter-induced oxidative stress and inflammatory responses in mouse skin by superoxide generation inhibitor 1'-acetoxychavicol acetate. Cancer Research 58, 4832-4839.

Nores, M.M., Courreges, M.C., Benencia, F., Coulombie, F.C., 1997. Immunomodulatory activity of *Cedrela lilloi* and *Trichilia elegans* aqueous leaf extracts. Journal of Ethnopharmacology 55, 99-106.

Noro,T., Sekiya,T., Katoh,M., Oda,Y., Miyase,T., Kuroyanagi,M., Ueno,A., Fukushima, S., 1988. Inhibitors of xanthine oxidase from *Alpinia galanga*. Chemistry and Pharmacology Bulletin 36, 244–248.

Rajan, S., Sethuraman, M., Mukherjee, K., 2002. Ethnobiology of the Nilgiri Hills, India. Phytotherapy Research 16, 98-116.

Rukachaisirikul, T., Prabpai, S., Champung, P., Suksamrarn, A., 2002. Chabamide, a novel piperine dimer from stems of *Piper chaba*. Planta Medica 68, 853-855.

Suzuki, Y., Koyanagi, Y., Tanaka, Y., Murakami, T., Misawa, N., Maeda, N., Kimura, T., Shida, H., Hoxie, J.A., O'Brian, W.A., Yamamoto, N., 1999. Determinant in human immunodeficiency virus type 1 for efficient replication under cytokine-induced CD4+ T helper 1(Th1) and Th2 type conditions. Journal of Virology 73, 316-324.

Tanaka, T., Shimizu, M., Kohno, H., Yoshitani, S., Tsukio, Y., Murakami, A., Safitri, R., Takahashi, D., Yamamoto, K., Koshimizu, K., Ohigashi H., Mori, H., 2001. Chemoprevention of azoxymethane-induced rat aberrant crypt foci by dietary zerumbone isolated from *Zingiber zerumbet*. Life Science 69, 1935–1945.

Testi, R., Phillips, J.H., Lanier, L.L., 1989. Leu23 induction as an early marker of functional CD3/T cell antigen receptor triggering: requirement for receptor cross-linking, prolonged elevation of intracellular (Ca^{++}) and stimulation of protein kinase. Journal of Immunology 142, 1854-1860.

Tewtrakul,S., Subhadhirasakul, S., Kummee, S., 2003. HIV-1 protease inhibitory effects of medicinal plants used as self-medication by AIDS patients. Songklanakarin Journal of Science and Technology 25, 239-243.

Unger, K.K.,1990. Packing and stationary phases in chromatographic techniques, Chromatographic Science Series. Marcel Dekker, New York.

Van der Nat, J.M., Klerx, J.P.A.M., Van Dijk, H., de Silva, K.T.D., Labadie, R.P., 1987. Immunomodulatory activity of an aqueous extract of *Azadirachta indica* stem bark. Journal of Ethnopharmacology 19, 125-131.

Vuddhakul, V., Seow, W.K., McCormack, J.G., Thong, Y.H., 1989. Direct modulation of human neutrophil behaviour by *Candida albicans*. International. Archive Allergy Applied Immunology 90, 291-296.

Wagner, H., 1990. Search for plant derived natural products with immunomodulatory activity. Pure and Applied Chemistry 2, 1217-1222.

Wiart, C., Mogana, S., Khalifah, S., Maha, M., Ismail, S., Buckle, M., Narayana, A.K., Sulaiman, M., 2004. Antimicrobial screening of plants used for traditional medicine in the state of Perak, Peninsular Malaysia. Fitoterapia 75, 68-73.

Yang, X., Eilerman, R.G., 1999. Pungent principal of *Alpinia galanga* (L.) swartz and its applications. Journal of Agricultural and Food Chemistry 47, 1657-1662.

Table 1 Plant names and their parts used for the extracts tested for immunomodulatory activities

Botanical name	Family	Part used
Acanthus ebracteatus Vahl	Acanthaceae	Leaf and stem
Alpinia galanga (L.) Willd.	Zingiberaceae	Rhizome
Barleria lupulina Lindl.	Acanthaceae	Leaf
Barleria lupulina Lindl.	Acanthaceae	Stem
Boesenbergia pandurata (Roxb.) Schltr.	Zingiberaceae	Rhizome
Coccinia grandis (L.) Voigt	Cucurbitaceae	Leaf
Eclipta prostrata (L.) L.	Asteraceae	Whole plant
Murraya paniculata (L.) Jack	Rutaceae	Leaf
Piper betle L.	Piperaceae	Leaf
Piper chaba Hunter	Piperaceae	Fruit
Spilanthes acmella (L.) Murray	Asteraceae	Whole plant
Zingiber zerumbet (L.) Roscoe ex Sm.	Zingiberaceae	Rhizome

Table 2 CD69 expression on lymphocytes after stimulation with various concentrations of PHA, SEB and PMA

Treatment	CD69 expression increase(%)
Control (non-stimulated)	4.5
PHA 5 μg/ml	52.7
PHA 10 μg/ml	62.4
PHA 25 μg/ml	65.1
SEB 5 μg/ml	24.7
SEB 10 μg/ml	25.7
SEB 25 μg/ml	26.5
PMA 5 ng/ml	29.5
PMA 10 ng/ml	35.4
PMA 25 ng/ml	48.9

500 μl of heparinized whole blood stimulated with various concentrations of PHA, SEB or PMA for 4 h at 37° C

PHA = phytohemagglutinin

SEB = staphylococcal enterotoxin B

PMA = phorbal 12-myristate acetate

Table 3 Effect of 36 crude extracts from 11 medicinal plants on CD69 expression on lymphocytes

Botanical name	% stimulation of CD69 expression		
	Extraction method		
	$CHCl_3$	MeOH	H_2O
Acanthus ebracteatus	0	0.5	9.0
Alpinia galanga	19.6*	2.9	3.8
Barleria lupulina (leaf)	1.7	0.7	1.5
Barleria lupulina (stem)	0.8	0.6	8.5
Boesenbergia pandurata	21.0*	4.2	1.9
Coccinia grandis	0.6	1.0	2.3
Eclipta prostrata	0	0.9	4.0
Murraya paniculata	0.2	0.5	2.2
Piper betle	0.7	0.3	2.8
Piper chaba	29.0*	1.5	4.5
Spilanthes acmella	0	0.5	2.5
Zingiber zerumbet	31.6*	5.8	2.3
Non-stimulated	6.2		
PHA stimulated	45.5		

Human whole blood was incubated with tested extracts at a final concentration of 1,000 µg/ml for 4h, stained with fluorescent conjugated antibodies CD4 perCP and CD69 PE and CD69 was investigated by flow cytometry.

PHA was used as positive control.

* significantly different from non-stimulated

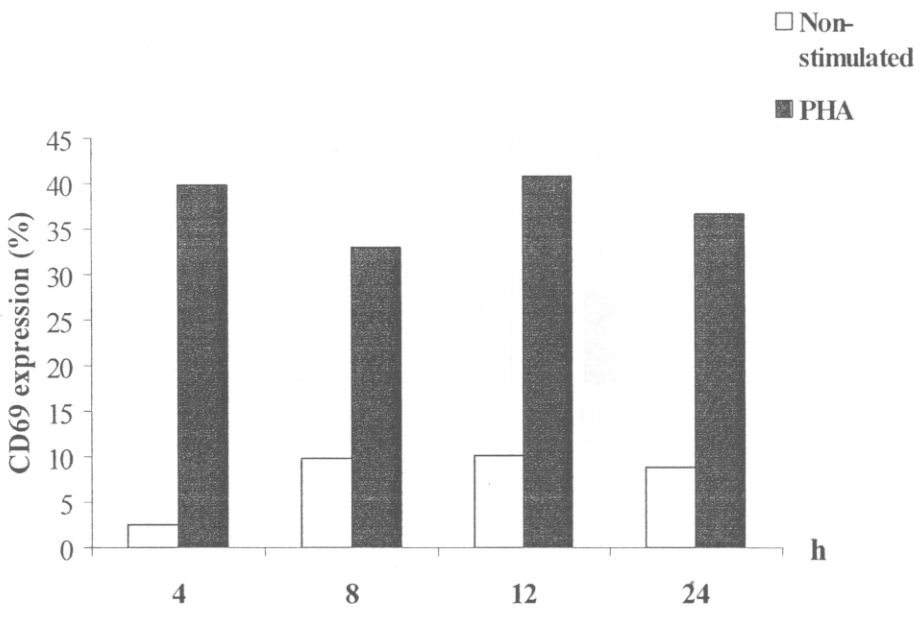

Fig. 1. CD69 expression on lymphocytes at various time incubation.

Human whole blood was incubated with PHA at the final concentration of 5 μg/ml, for either 4, 8, 12 and 24h, stained with fluorescent conjugated antibodies CD4 perCP and CD69 PE and CD69 expression was determined by flow cytometry.

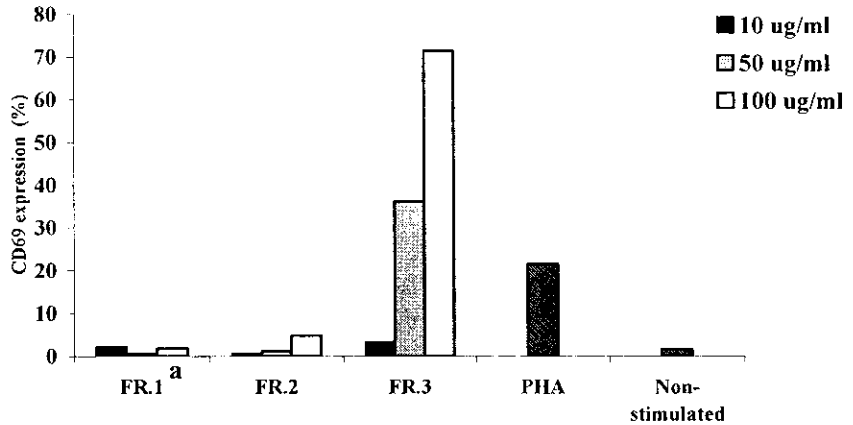

Fig. 2. CD69 expression on lymphocytes after treatment with 3 fractions of *Boesenbergia pandurata*

Human whole blood was incubated with tested fractions at final concentration of 10, 50 and 100 µg/ml for 4h, stained with fluorescent conjugated antibodies CD4 perCP and CD69 PE and CD69 was investigated by flow cytometry.

PHA was used as positive control.

[a] Fraction 1

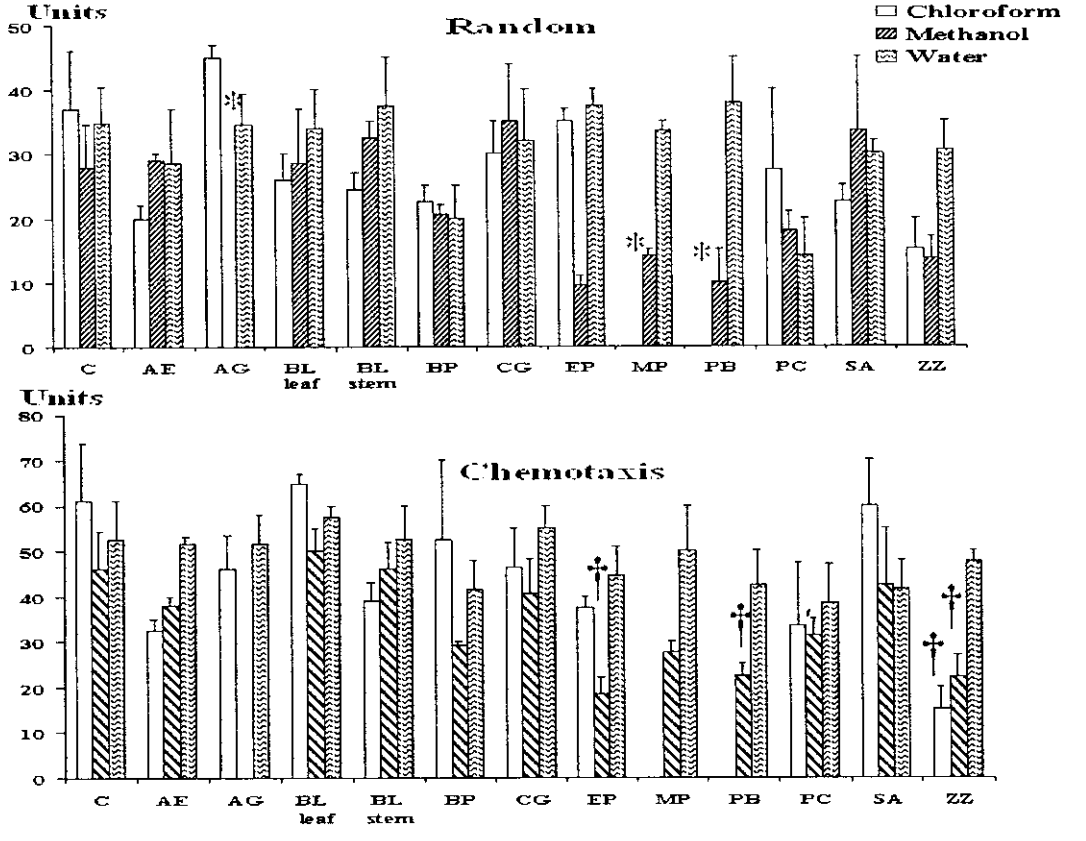

Fig. 3. Effect of crude extracts on polymorphonuclear migration rates.

Polymorphonuclear cells were incubated with test extracts at the final concentration of 100 ug/ml for 2 h and their migration was investigated.

AE = *Acanthus ebracteatus,* AG = *Alpina galanga,* BL leaf = *Barleria lupulina,* BL stem = *Barleria lupulina,* BP = *Boesenbergia pandurata,* CG = *Coccinia grandis,* EP = *Eclipta prostrata,* MP = *Murraya paniculata,* PB = *Piper betle,* PC = *Piper chaba,* SA = *Spilanthes acmella,* ZZ = *Zingiber zerumbet*

* cell lysis ✝ suppression