Immunomodulatory Activities of *Artocarpus incisus*, Linn. F. Extract

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ABSTRACT

The immunomodulatory effects of *Artocarpus incisus* heartwood extract on macrophage and splenocytes of ICR mouse were studied. The phagocytic activity of peritoneal macrophages and splenocyte proliferation in the absence and presence of mitogens (phytohemagglutinin, concanavalin A, lipopolysaccharide and pokeweed mitogen) were assayed. The extract effected to the enhancement of lysosomal enzyme activity but had no effect on the oxidative burst reduction on phagocytosis. Antiproliferative activity of spleen T- and B-lymphocytes was also presented. The extract with PWM gave the maximum suppression of B-lymphocyte proliferation, suggesting specificity towards T cell dependent pathway the same as PWM. These results may prove the folklore remedy of this plant relating to immune system.

Keywords: *Artocarpus incisus*, ICR mouse, phagocytosis, antiproliferative activity, immune system

INTRODUCTION

Breadfruit Tree *(Artocarpus incisus)* is a plant in the family Moraceae and is grown for fruits. It is believed to be native to a vast area extending from New Guinea through the Indo-Malayan Archipelago to Western Micronesia. It has been widely spread in the Pacific area by migrating Polynesians. In Trinidad and the Bahamas, a decoction of the breadfruit leaf is believed to lower blood pressure and relieve asthma. Crushed leaves are applied on the tongue as a treatment for thrush. Ashes of burned leaves are used on skin infections. Toasted flowers are rubbed on the gums around an aching tooth. The latex is used on skin diseases and is bandaged on the spine to relieve sciatica. Diluted latex is taken internally to overcome diarrhea. Moreover, a powder of roasted leaves is employed as a remedy for enlarged spleen (Morton 1987). The extract from the heartwood of *A. incisus* is a rich source of flavones with isoprenoids substituent (Kuniyoshi et al. 1998). Varieties of biological activity of *A. incisus* extract were reported. Isoprenoid-substituted flavonoids and stilbenes isolated from *A. incisus* possess inhibitory effects on melanin biosynthesis (Shimizu et al. 1998). They also inhibit 5α-reductase activity (Shimizu et al. 2000), in the amino acid transport system in Lepidoptera (Parenti et al. 1998), arachidonate 5-lipoxygenase and mouse TNF-a release (Nomura et al. 1998). Their cytotoxicity against cancer cells, anti-platelet aggregation activity in rabbit platelet suspensions, and antibacterial activities against cariogenic bacteria have also been studied (Nomura et al. 1998). There were some reports of plants in the same genus, which had the immunopotentiating properties *in vitro* and/or *in vivo*. *A. integrifolia* induced IL-2 production by macrophages and switch from type 2 to type 1 cell-mediated immunity *against*

Leishmania major antigens, resulting in Balb/c mice resistance to infection (Ademilson et al. 2001). The cytotoxicity *in vitro* of flavones (artelastocarpin and carpelastofuran) and prenyl flavonoids (artelastin) from *A. elasticus* against the three cancer cell lines (Cidade et al. 2001) and artoindonesianin L. from *A. rotunda* against murine P388 leukemia cells (Suhartati et al. 2001) were also presented. However, the immunomodulating activity of *A. incisus* has never been performed. This study investigated the *in vitro* immunomodulating activity of *A. incisus* on ICR mouse macrophage phagocytosis and splenocyte proliferation.

METHODOLOGY

Plant materials

The heartwood of *A. incisus* was collected in September 2003 from Phitsanulok, Thailand. The voucher specimens (no. AI092003) were authenticated by the botanist in Department of Pharmacognocy and preserved at Department of Pharmaceutical Technology, Naresuan University, Thailand.

Preparation of the extracts

The heartwood of *A. incisus* was chipped and milled to pass a #40 screen. The air-dried milled heartwood was macerated for 10 days in with diethyl ether at room temperature (25$\pm$2°C) and the extract was concentrated under reduced pressure to dryness. The yield of the extract was 0.9% (w/w of the dried woods) and was kept in a tight container at 4°C until use.

Animals

ICR mice, 4-5 weeks old, were used in all experiments. The animals were purchased from National Laboratory Animal Center, Mahidol University, Bangkok, Thailand. The animals were housed under standard conditions at 25$\pm$2°C and fed with standard pellets and tap water.

Preparation of peritoneal mouse macrophages

Peritoneal mouse macrophages were prepared as previously described (Manosroi et al. 2003). Fetal calf serum (FCS, Biochem KG, Germany) was injected intraperitoneally into mice as a stimulant to elicit peritoneal macrophages. Three days after injection, the resulting peritoneal cells were harvested by peritoneal lavage with RPMI-1640 (Sigma-Aldrich, Germany) supplemented with 10% heat-inactivated FCS, 50 μM 2-mercaptoethanol (Pharmacia, Sweden), 100 I.E. penicillin, 100 μg streptomycin and 0.25 μg/ml amphotericin B (Sigma-Aldrich, Germany). The exudate was centrifuged at 1000 rpm, 25 $^{\circ}$C for 20 min. The erythrocytes in the cell pellets were lysed by hypotonic solution (0.2% NaCl). Isotonicity was restored with 1.6% NaCl solution. Cell suspension was centrifuged and the cells were washed twice and re-suspended in complete RPMI-1640. The cell number was adjusted to 1×10^6 cell/ml. The trypan-blue dye exclusion techniques were used to determine the viability of macrophages.

Preparation of mouse splenocytes

Mouse splenocytes were prepared as previously described (Manosroi et al. 2003). Mice were sacrificed and their spleens were removed aseptically. The cell suspension was prepared by means of loose potter and flushing. After centrifugation at 1000 rpm for 10 min at 25°C, erythrocytes were lysed by hypotonic solution and the cell pellets were washed twice with RPMI-1640. The cells were re-suspended in complete RPMI medium and the cell number was adjusted to 2×10^6 cell/ml. The viability of splenocytes was determined by the trypan-blue dye exclusion technique.

In vitro phagocytic assay on nitroblue tetrazolium (NBT) reduction

The NBT reduction assay was carried out according to the method previously described (Rainard 1986). Briefly, 20 µl of the plant extracts was added to 20 µl of the cells (1×10^6 cells/ml) and 40 µl of RPMI in 96-well plate (Nunc®, USA). The final concentrations of the plant extracts were 0.05, 0.25, 0.50, 0.75 and 1 mg/ml per well. After incubation for 24 h at 37°C in humidified 5% CO_2, 20 µl of zymozan A (Sigma-Aldrich, Germany) suspension (5×10^7 particles/ml) and 20 µl of NBT (Sigma-Aldrich, Germany) solution (1.5 mg/ml) in phosphate buffer saline (PBS) were added. Dimethylsulfoxide (DMSO) which was adjusted to 0.1% (v/v) was used as control. The culture was incubated for further 60 min and the adherent macrophages were rinsed vigorously with RPMI 1640 medium. The supernatant was pipetted off and washed four times with 200 µl methanol (Analar, England) to eliminate the unreduced NBT dye. The cell pellets were air dried. An amount of 120 µl of 2 M KOH and 140 µl of DMSO was added consecutively. The absorbance of the turquoise blue solution was measured at 570 nm by a well reader (Seikagaku SK601, Japan). The % NBT reduction was calculated by the following equation:

% NBT reduction = [(OD sample-OD control)/OD control]×100

In vitro phagocytic assay on cellular lysosomal enzyme activity

The cellular lysosomal enzyme activity determined by acid phosphatase in macrophages was assayed as previously described (Suzuki et al., 1988). Briefly, 20 µl of the plant extracts was added to 20 µl of macrophage suspension ($1x10^6$ cells/ml) and 40 µl of RPMI medium in 96-well plates at 37°C in humidified 5% CO_2 incubator for 24 h. The final concentrations of the plant extracts in each well were 0.05, 0.25, 0.50, 0.75 and 1 mg/ml. The medium was discarded and the macrophage monolayer in each well was solubilized with 20 µl of 0.1% Triton X-100 (Pharmacia, Sweden). An amount of 100 µl of 10 mM *p*-nitrophenyl phosphate (*p*-NPP) (Sigma-Aldrich, Germany) solution and 50 µl of 0.1 M citrate buffer (pH 5.0) were added. The culture was further incubated for 30 min. An amount of 150 µ l of 0.2 M borate buffer (pH 9.8) was added to terminate the reaction and the absorbance at 405 nm was measured. The %lysosomal enzyme activity was calculated by the following equation:

% Lysosomal enzyme activity = [(OD sample-OD control)/OD control]×100

In vitro proliferation assay in mouse splenocytes

The proliferation assay of lymphocytes was tested according to 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Mosmann 1983). Briefly, 20 µl of various concentrations (0.05, 0.25, 0.50, 0.75 and 1 mg/ml) of extracts was added to 20 µl of cell suspension (1×10^6 cells/ml) and 40 µl of RPMI in a 96-well plate. Proliferation of cells in the absence and presence of mitogens was investigated. The predetermined optimum dose of phytohemagglutinin (PHA), concanavalin A (con A), lipopolysaccharide (LPS) and pokeweed mitogen (PWM) at 5 µg/ml was used. After incubation at 37°C in humidified 5% CO_2 for 48 h, 20 µl of MTT (Sigma-Aldrich, Germany) (5 mg/ml) in PBS and 40 µl of complete RPMI were added. The culture medium was discarded by aspiration and added with 100 µl of 0.04 M HCl in isopropanol (Lab-Scan Ltd., Ireland) to lyse cells. Then, 100 µl of distilled water was added to dilute the solution and the absorbance was measured at 570 nm. The %inhibition of mitogen induced splenocyte proliferation was calculated by the following equation:

$$\% \text{ Inhibition} = [1-(\text{OD sample} / \text{OD mitogen})]\times100$$

Statistical analysis

All experiments were performed in triplicate and the results were expressed as mean±S.E. Student's *t*-test was used to analyze statistical significance of the differences between the control and the treated values.

RESULTS

Phagocytic activity assay in mouse macrophages

Effects of *A. incisus* extract on the NBT dye reduction and lysosomal enzyme activity response of macrophages were shown in Fig. 1. *A. incisus* extract ranging from 0.05 to 1 mg/ml increased the lysosomal enzyme activity in a non-dose dependent manner with the maximum effect of approximately 75% at 0.5 mg/ml. However, *A. incisus* did not have any effects on the NBT dye reduction within this tested concentration ranges.

In vitro proliferation assay in mouse splenocytes

Effects of *A. incisus* extract (0.50–1 mg/ml) on the proliferative response of splenocytes without or with mitogens (PHA, Con A, LPS, PWM) was demonstrated (Fig. 2). All tested concentrations of the extract without mitogens showed similar proliferation activity to the control (data not shown). A significant inhibition of splenocyte proliferation was observed in all concentrations of extract with mitogens. *A. incisus* extract at 0.75 mg/ml with PHA or Con A diminished the splenocyte proliferation with the maximum inhibition of 16 and 24%, respectively, while *A. incisus* at 1 mg/ml with LPS and at 0.25 mg/ml with PWM caused 37% and 46% inhibition of proliferation, respectively.

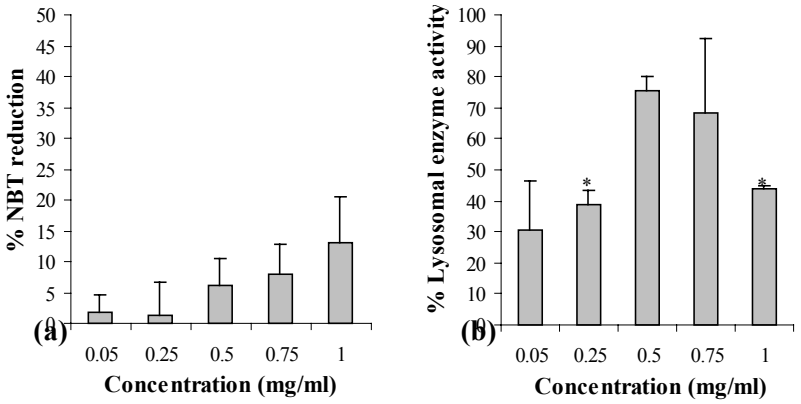

Figure 1. Effects of different concentrations of *A. incisus* extract on phagocytosis response of ICR mouse macrophages: (a) NBT dye reduction, (b) Lysosomal enzyme activity. Each value represents the mean±S.E. of triplicate comparing to the control;
**P* < 0.01

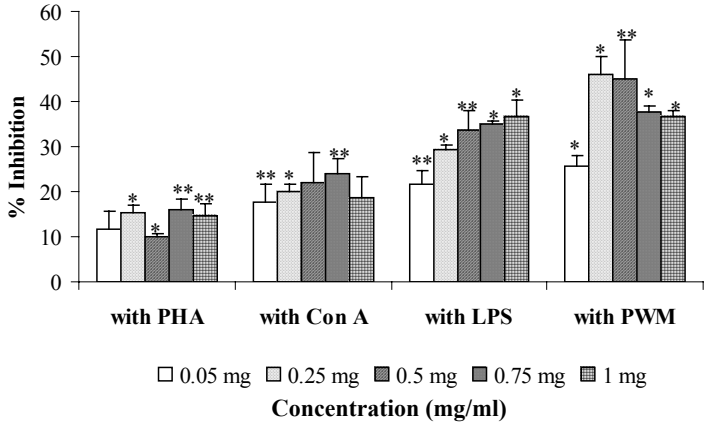

Figure 2. Effects of different concentrations of *A. incisus* extract on proliferation response of ICR mouse splenocytes with PHA, Con A, LPS and PWM (5 μg/ml). Each value (% inhibition) represents the mean±S.E. of triplicate comparing to each mitogen; **P* < 0.01, ***P* < 0.05.

DISCUSSION AND CONCLUSION

This study indicated that extract of *A. incisus*, a plant that has been used in various remedies including the treatment for infection and enlarged spleen, exerted *in vitro* immunomodulatory activities on ICR mice immune system.

It has been well known that macrophages play an important role in the defense mechanism against host infection and the killing tumor cells (Kang et al. 2002). The modulation of macrophages functions by various biological response

modifiers is an area of active interest for treatment of infection, cancer chemotherapy and immune deficiency disorders. The higher reduction in NBT assay represented higher activity of oxidase enzyme, reflecting the stimulation of phagocytes in proportional to intracellular killing (Rainard 1986). For lysosomal enzyme activity, transformation of *p*-nitrophenyl phosphate (*p*-NPP) to the colored compound by the acid phosphatase of the stimulated macrophages was correlated to degranulation in phagocytosis (Suzuki et al. 1990). Our results showed that *A. incisus* extract exhibited a strong lysosomal enzyme activity but had no effect on NBT dye reduction. Therefore, *A. incisus* extract may affect phagocytic activity, mainly having influences on acid phosphatase production in degranulation process.

Colorimetric MTT assay was used for splenocyte proliferation since the cleavage of MTT has several desirable properties for assaying cell survival and proliferation. MTT is cleaved by all living, metabolically active cells and the amount of MTT formazan generated is directly proportional to the cell number (Mosmann 1983). PHA and Con A were used for activation of different subtypes of T cell proliferation. LPS was used for stimulation of T cell independent B cell proliferation whereas PWM was for activation of T cell dependent B cell proliferation. The presence of mitogens in the system can postulate the possible mechanism of the extracts (Nakamura et al. 1986).

All concentration ranges of *A. incisus* extract with mitogens exerted the inhibitory effect on splenocyte proliferation whereas the extract without mitogens did not show this effect. The extract may have an antiproliferative effect towards T- and B-cells. In the optimum concentrations of PHA or Con A (5 μg/ml), the extract had weak suppression of T lymphocytes whereas with the optimum concentrations of LPS and PWM (5 μg/ml), the extract showed a strong inhibitory activity of B-lymphocytes. The maximum suppressive activity induced by PWM was approximately 10% more than by LPS induction. Hence, this suggests that *A. incisus* extract had inhibitory effect to B cell proliferation through the T cell dependent pathway the same as PWM, more than through the T cell independent mechanism the same as LPS.

Flavonoids, natural polyphenol compounds, were known to have anti-inflammatory and immunomodulatory activities. Differences in sensitivity to T- and B-mitogenic stimuli have already been described for flavonoids. Some flavonoids may affect T-cell proliferation to a greater extent than B-cell proliferation or may affect two populations equally (Hirano et al. 1989; Namgoong et al. 1993; You et al 1998). In contrast, several biflavonoids such as ginkgetin, isoginkgetin, ochnaflavone, cryptomerin B and isocryptomerin showed the suppressive activity against lymphocyte proliferation induced by Con A or LPS. Apigenin (flavone) and quercetin (flavonol) were suppressive against Con A-induced lymphocyte proliferation, but not against LPS-induced lymphocyte proliferation at the same concentration range (Lee et al. 1995).

Wood and bark of *Artocarpus* spp. are rich in flavonoids and their derivatives (Baron and Ibraham 1996). The immunoregulation activity of *A. incisus* wood extract might be from flavonoids. This was supported by the similar results of other plants of the same genus. Artelastin, prenylated flavones isolated from *A. elasticus*

exhibited a dose-dependent suppression effect on the mitogen response of human lymphocytes to PHA (Cerqueira et al. 2003).

The present results demonstrated the stimulation of macrophage function. The extract per se does not have immunoregulating activity, but in the presence of optimum concentrations of mitogen, a significant antiproliferative activity of lymphocyte was observed. The mechanism whereby the extract affected the mouse immune system remained unclear. The results from this study may prove the folklore remedies of *A. incisus* for the treatment of infection, inflammation and the enlarged spleen. Further work to isolate the active constituent and to clarify its mode of action will be in the next report.

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