The Effects of Ayurved Siriraj Wattana Recipe on Splenocytes in Wistar Rat

Sunisa Duangsa-ard, M.Sc.*, Adisak Wongkajornsilp, M.D., Ph.D.*, Pravit Akarasereenont, M.D., Ph.D.*,, Sukit Huabprasert, M.Sc.*, Tanta Chongputtharaksa**, Tawee Laohapand, M.D.****

**Department of Pharmacology, **Center of Applied Thai Traditional Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand.*

ABSTRACT

Wattana Ayurved Siriraj herbal formulary® has been used for decades and is quoted to carry anti-ageing action. Here, we have investigated the immunological efficacy and the safety for the consumption of Ayurved Siriraj Watana Recipe in Wistar rats. Rats were orally administered with Ayurved Siriraj Watana Recipe at a dose range of 0, 100, 300 and 1,000 mg/kg Body Weight (BW) for 14 days. After the treatments, splenocytes were isolated from these rats and examined for cell proliferation and NK cell activities. We found that the ingestion of Ayurved Siriraj Watana Recipe was not toxic to Wistar rats in all doses based on general gross and microscopic examinations. The recipe could not drive the proliferation of rat splenocytes in a dose-dependent manner. The ingested Ayurved Siriraj Watana Recipe also could not drive the NK cell cytotoxicity. Both the proliferative and NK cell assay revealed unfavorable response from rats ingesting 300 mg/kg BW AVS073. In summary, the ingestion of Ayurved Siriraj Watana Recipe in Wistar rats was safe with the studied dose. The use of Wistar rats for immunological screening of Ayurved Siriraj Watana Recipe or others is not suitable, since conventional mitogens also failed to stimulate their splenocytes. Other established animal models (e.g., BALB/c mice) would be more relevant.

Keywords: Wattana Ayurved Siriraj, splenocyte, immunostimulation

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INTRODUCTION

The improving conditions of longevity have been a prominent health concern with a sizable amount of expenditure. The components of "longevity/anti-ageing" have not been clearly defined. Several pharmaceuticals were claimed to possess anti-ageing activities based on anti-oxidants[1] and anti-inflammatories.[2] These activities might have been inherent to some traditional anti-ageing recipes that have been traditionally prescribed for longevity. Ayurved Siriraj Watana Recipe (AVS073) has long been used for healing in Thai traditional medicine treatment for health promotion, appetite inducement and retardation of health degeneration (anti-aging). The formula comprises *Aeglemarmelos* (Linn.), *Boesenbergia rotunda* (L.), *Caesalpiniasappan* L., *Carthamustinctorius* L., *Cinnamomumsiamense* Craib., *Citrus sinensis* L.Osbeck, *Cladogynosorientalis*, *Cryptolepisbuchanani* Roem. & Schult., *Cyperusrotundus* L., *Derris scandens* Benth., *Drypetesroxburghii* Wall., *Ferula assafoetida* Linn., *Saussureealappa* Clark., *Ligusticumsinense* Oliv., *Mallotusrepandus* (Willd.), *Piper nigrum* Linn., *Terminaliachebula* Retz., and *Tinosporacrispa*. We scrutinized the recipe for immunomodulatory activity, a major component of longevity. The putative immuno-modulatory effects of this recipe were evaluated in Wistar rats ingesting the recipe for 14 days. We proposed that the ingested recipe would improve the immuno-stimulatory activity of innate immune response that indirectly serves its anti-cancer action. The assay for NK cell activity in isolated splenocytes was chosen to demonstrate this action. The outcomes from this study would provide the safety profile of AVS073 and confirm the presence of immuno-stimulatory effect that would prompt the determination of efficacious dose to humans.

MATERIALS AND METHODS

Preparation of Ayurved Siriraj Watana Recipe Powder

The Ayurved Siriraj Watana Recipe was manufactured as powder under GMP Guidelines by the Center of Applied Thai Traditional Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University. Herbal raw materials

Correspondence to: Adisak Wongkajornsilp
E-mail: adisak.won@mahidol.ac.th
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were authenticated, washed and dried in a hot air oven in accordance with the Ayurved Siriraj Watana Recipe Master Formula. It was validated with chemical fingerprint usingultra-performanceliquid chromatography. The preparation contains gallic acid, caffeic acid, and either p-coumaric acid or ferulicacid (Fig 1). It was kept dried at 25°C and protected from light. The recommended daily consumption for humans was multiplied by 7 for animal study,[3] resulting in the daily dose range of 100-1,000 mg/kg body weight.

Animal studies

Wistar rats at the age of 6-8 weeks with 25-30 g body weight were supplied by the National Laboratory Animal Centre, Mahidol University. They were weighed daily and observed for mortality. The study protocol had been approved by Siriraj Animal Care and Use Committee (SI-ACUC). The minimum of 4 animals/group was allocated according to the guideline.[4] They were housed in individual ventilated cages (IVC, Technicplast, Italy) at 4 rats per cage, maintained at 23 ± 2°C, 40-70 % humidity with a 12:12 h light:dark cycle. Animals had free access to laboratory chow (C.P. feed 082, Thailand) and distilled water *ad libitum*. The rats were fed for 14 days with 100, 300 and 1,000 mg/kg AVS073 in 3 mL sterile distilled water. On the final day (24 h after the last dose), animals were sacrificed by cervical dislocation and dissected for spleen.

Splenocytes isolation

The spleens were aseptically excised and placed in 15-mL centrifuge tube containing 10 mL serum-free RPMI-1640. The cell suspension was prepared by grinding the spleen using a syringe with 21 gauge needle in 10 mL RPMI-1640, laid over IsoPrep® (Robbins Scientific, Canada) density gradient and centrifuged at 900 × g for 30 min at 20°C.[5,6] The ring containing lymphocytes was collected and washed twice with RPMI-1640 containing 10% FBS by centrifugating at 900 × g, 20°C for 10 min. The viability of the isolated splenocyte determined by trypan blue exclusion test was greater than 95%. Splenocytes were finally washed and resuspended in RPMI-1640, 100 U/mL penicillin and 100 µg/mL streptomycin.

Preparation of target cells (YAC-1)

YAC-1 (CLS Cell Lines Service, Eppelheim, Germany) was used as cellular targets for NK cells.[5,7] They were maintained in RPMI-1640,10% FBS, 100 IU/mL penicillin, 100 µg/mL streptomycin at 37°C in 5% CO_2.

Splenocyte proliferation assay

Aliquots (100 µL) of splenocytes in RPMI-1640 with either 2% or 10% FBS[8] were plated at a density of 1×105 cells per well in a 96-well plate. The splenocytes were treated with either lipopolysaccharide (LPS, 5 µg/mL) or concanavalin A (ConA, 5 µg/mL) to magnify cell numbers. The cells were incubated at 37°C and 5% CO_2 for 72 h. MTT in PBS (10 µL of 5 mg/mL) was added to each well (containing 100 µL cell suspension) and incubated at 37°C, 5% CO_2 for 4 h. Finally,100 µL of 0.04 N HCl in isopropanol was added to each well. The formation of MTT-formazan was measured at 570 nm using an ELISA microplate reader (SpectraMax M5, Molecular Devices). The absorbance was calculated as the percentage of the control as follows:

$$\text{Proliferation (\%)} = \frac{\text{OD (sample)} - \text{OD (background)}}{\text{OD (control)} - \text{OD (background)}} \times 100$$

The sample OD was the observed absorbance from the well containing the treated sample. The control OD was the absorbance from the well containing unstimulated cells (no mitogennor AVS073). The background OD was the absorbance from well containing only the corresponding supernatant without any cellular component.

Natural Killer (NK) cell cytotoxic assay

Splenocytes were evaluated for natural killer (NK) cell cytotoxic activity using an ELISA microplatereader[9]. YAC-1 (40 µL of 1×10^4 cells/ well) was seeded in triplicate onto the flat-bottom 96-well plate. Subsequently, 40 µL of effector cells was added to each well in triplicate, resulting in effector: target (E:T) ratios of 3.125: 1, 6.25: 1, 12.5: 1 and 25:1.[7,10] After 3 h at 37°C in 5% CO_2, 20 µL of propidium iodide solution (60 µg/mL in serum-free RPMI1640) was added. Fluorescence was measured using a 482 nm exciter filter and 630 nm emission filter. The mean fluorescence unit was obtained from triplicate wells. The cytotoxicity was calculated as follows:

$$\text{Cytotoxicity (\%)} = \frac{\text{Test units} - \text{spontaneous units of target and effector}}{\text{Maximal units of target} - \text{spontaneous units of target}} \times 100$$

The maximal units of target came from thefluorescence intensity of well containing 100% lysis of target cells (YAC-1). The 100% lysis of target cells was accomplished by adding 20 µL/well of 0.04N HCl in isopropanol. The spontaneous unit of target came from the fluorescent intensity of wells containing only untreated target cells. The test unit came from the fluorescence intensity emanated from wells containing the mixture of effector (E) and target (T) at different E:T ratios (3.125: 1, 6.25: 1, 12.5: 1 and 25: 1). The spontaneous unit of target and effector came from the fluorescent intensity which emanated from wells containing the only corresponding density of effector cells plus those from the target cells alone.

Statistical analysis

The data were ere analyzed using GraphPad Prism version 5.02 and expressed as mean ± SEM. Either one-way ANOVA with Dunnett's test or two-way ANOVA with Bonferroni test was used to analyze statistical significance of the differences between the controls and the treated samples. Data were considered statistically significant if $p < 0.05$.

RESULTS

The physical and behavioral appearances of rats ingesting Ayurved Siriraj Watana Recipe

The study displayed no toxicity of the AVS073 in Wistar rats at doses ranging from 100-1,000 mg/kg body weight. Therefore, AVS073 is relatively safe as determined by the given toxicity studies. There was no evidence of toxicity as monitored by general behavior change, mortality, or change in gross appearance of internal organs. Rats fed with AVS073 for 14 days had no difference in body weight from untreated rats throughout the study (data not shown).

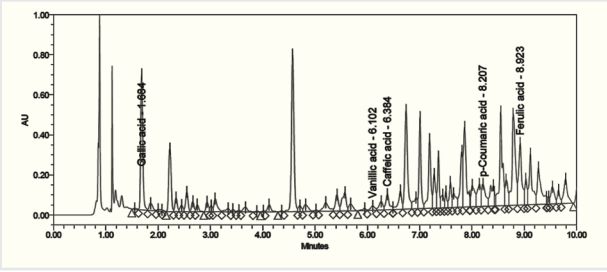

Fig 1. The UPLC chromatogram of AVS073. The positions of the peaks that corresponded to those of the contemporary runningstandards were marked accordingly.

The proliferation of splenocytes in response to Ayurved Siriraj Watana Recipe

The in vivo experiments were conducted to determine immuno-stimulating effects occurring in Wistar rats. The toxicity was evaluated after 14-day oral administration. To avoid the growth promoting activity from 10% FBS that could mask the corresponding activity from AVS073, a low concentration (2%) of FBS was employed to minimize this activity while still sufficiently maintaining splenocyte viability. At high-levels of (10%, Fig 2) FBS, the splenocytes isolated from these treated rats could not be driven for proliferation over those from the untreated rats (0 mg/kg BW). AVS073at 300 mg/kg BW, but not at 1,000 mg/kg BW, suppressed the splenocyte proliferation. There was no enhancement of splenocyte proliferation from any given dose. The addition of LPS provided slightly, but insignificant, proliferative activity at all doses of AVS073, except at 300 mg/kg BW where there was a significant improvement. The LPS treatment generally provided better proliferative activity in all conditions over those without LPS. The addition of ConA, instead of providing mitogenic activity, compromised the splenocyte viability. However, AVS073 at 300 mg/kg prevented the suppressive activity of ConA. At low-levels of (2%, Fig 3) FBS, the addition of LPS did not exhibit any statistically significant difference from those without mitogens. However, ConA significantly suppressed the splenocyte proliferative activity in almost all doses of AVS073 except at 1,000 mg/kg BW. Further

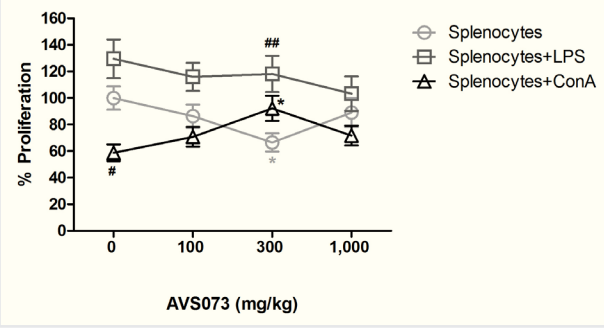

Fig 2. The 72-h incubation in growth media containing 10% FBS of isolated splenocytes from Wistar rat ingesting AVS073 at 100, 300, or 1,000 mg/kg BW (1×10^5 cells/well). Splenocytes in RPMI1640 with 10% FBS were stimulated either with LPS (5 µg/mL, □), ConA (5 µg/mL, △), or none (○) throughout the incubation. The proliferation of splenocytes was measured using MTT assay. Each value represented mean ± SEM of six independent experiments performed in triplicate (n=18). Statistically significant difference ($p < 0.05$) of samples treated with different dosages of AVS073 from those treated with the same mitogen but without AVS073 (0 mg/kg BW) is denoted as *. Statistically significant difference ($p < 0.05$, $p < 0.01$, or $p < 0.001$) among samples with the same level of AVS073, but different mitogens, from those without mitogen is denoted as #, ##, or ### respectively.

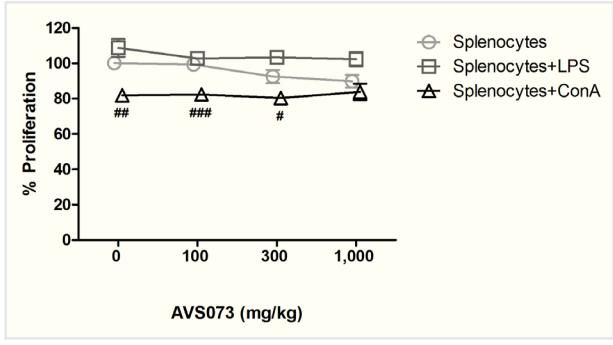

Fig 3. The 72-h incubation in growth media containing 2% FBS of isolated splenocytes from Wistar rat ingesting AVS073 at 100, 300, or 1,000 mg/kg BW (1×10^5 cells/well). Splenocytes in RPMI1640 with 2% FBS were stimulated either with LPS (5 µg/mL, □), ConA (5 µg/mL, △), or none (○) throughout the incubation. The proliferation of splenocytes was measured using MTT assay. Each value represented mean ± SEM of five independent experiments performed in triplicate (n=15). Statistically significant difference ($p < 0.05$) of samples treated with different dosages of AVS073 from those treated with the same mitogen but without AVS073 (0 mg/kg BW) is denoted as *. Statistically significant difference ($p < 0.05$, $p < 0.01$, or $p < 0.001$) among samples with the same level of AVS073, but different mitogens, from those without mitogen is denoted as #, ##, or ### respectively.

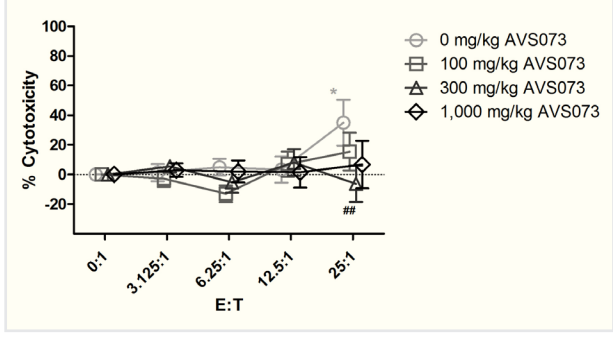

Fig 4. The anti-tumor cytotoxic activity of isolated splenocytes from Wistar rat ingesting AVS073 at 100, 300, or 1,000 mg/kg BW. The isolated splenocytes from rats ingesting different dosages of AVS073 served as effector cells. YAC-1 served as target cells. The PI uptake assay was chosen as the cytotoxicity test. Data were expressed as mean ± SEM of 6 rats / group. Difference with statistical significance ($p < 0.05$) within each treatment dosage from the corresponding control (E:T ratio= 0:1) is denoted as *. Difference with statistical significance ($p < 0.05$, $p < 0.01$, or $p < 0.001$) of each treatment dosage from those of the corresponding untreated splenocytes (0 mg/kg BW) is denoted as #, ##, or ### respectively.

analysis for the interaction between ConA and AVS073 at 1,000 mg/kg BW revealed the suppressive activity in all studied doses of ConA. The suppressive activity was further enhanced with the high dose AVS073 (data not shown).

The NK cell activity of splenocytes isolated from rats fed with Ayurved Siriraj Watana Recipe

Only splenocytes at the highest E:T ratio (25:1) from rats with no treatment with AVS073 provided significant cytotoxicity toward YAC-1 over the corresponding control (E:T ratio = 0:1, Fig 4). Smaller E:T ratios provided no significant difference. The treatment with AVS073 at all studied dosages, except at 300 mg/kg BW, did not yield any difference from the control.

DISCUSSION

We determined the effect of AVS073 on the proliferative response of lymphocytes after the stimulation with either one of the two different mitogens, ConA and LPS. Splenocytes from rats have been used for the study of immune-modulating activity of herbal recipes. AVS073 at all doses, except at 300 mg/kg BW, was relatively non-toxic to the splenocytes based on the proliferation assay at 10% FBS, but not at 2% FBS. It is possible that ingesting AVS073 at 300 mg/kg BW started exhibiting adverse effects toward the whole population of splenocytes, while at the higher dose (1,000 mg/kg BW), a selective subpopulation of splenocytes predominated and brought the overall improvement for the whole population. Likewise, FBS might exert its trophic effect toward certain subpopulations of splenocytes which resulted in some discrepancy between data from different levels of FBS. The splenocyte proliferation induced by B cell mitogen, LPS, was slightly enhanced, but T lymphocyte proliferation induced by ConA was not observed. Instead, ConA suppressed splenocyte proliferation, but was reversed in splenocytes from rats ingesting AVS073 up to 300 mg/kg BW, but not at the higher dose. It is concluded that AVS073 natively lacks the ability to drive rat overall splenocyte proliferation, but can neutralize the toxicity of ConA toward splenocyte proliferation at 300 mg/kg BW. A similar trend was observed at 2% FBS where AVS073 at 1,000 mg/kg BW could neutralize the adverse effect of ConA toward splenocyte proliferation. This study also suggested that ingesting AVS073at the studied doses is relatively safe for immune cells *in vivo* as 100% viability of freshly isolated splenocytes was achieved, but their proliferative function was compromised at 300 mg/kg BW.

Regarding the effect of AVS073 toward innate immune response, NK cell-mediated cytotoxic assay was performed using splenocytes isolated from rats ingesting AVS073 as effector cells and YAC-1 as target cells. We could not observe any significant enhancement of NK cell activity in any condition, but there was a decrease in the cytolytic activity from rats ingesting 300 mg/kg BW AVS073 at the E;T ratio of 25:1. It remains possible that higher E:T ratios would achieve significant activity over the control group. Wistar rat splenocytes themselves, as opposed to those from BALB/c mice,[9] might not be suitable for the immunological study since they failed to produce appreciable response to conventional mitogens. This might explain why there is only a single article employing rat splenocytes and YAC-1 cells in NK assay.[11]

In summary, the ingestion of Ayurved Siriraj Watana Recipe in Wistar rats was safe with the studied dose, except at 300 mg/kg BW, based on proliferation assay and NK cell assay. The use of Wistar rats for immunological screening of Ayurved Siriraj Watana Recipe or others is not suitable, since conventional mitogens also failed to stimulate their splenocytes. Other established animal models (e.g., BALB/c mice) would be more suitable for the elucidation of immunologic efficacy.

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