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Enhancing Th1 Cell Activities in Mice by Short-term Oral Administration of *Gynostemma pentaphyllum* Extracts

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ABSTRACT

Gynostemma pentaphyllum is a plant widely distributed in South-China, Korea, and Japan. It is taken as an herbal tea in China and some Asian countries. We had tried to use the extract of herb to intraperitoneally inject into mice for 5 consecutive days and found that it could enhance levels of IgG2a in serum and Th1 and Th2-associated cytokines in spleen cells cultured with Con A. We also asked the question of whether an oral administration of *G. pentaphyllum* could promote the production of serum IgG2a and Th cell-associate cytokines the same as intraperitoneal injection did. Our result showed that short-term oral administration of *G. pentaphyllum* could suppress the level of serum IgG1, and enhanced amounts of serum IgG2a and Th1-associated cytokines secretion from spleen cells cultures.

Key words: *Gynostemma pentaphyllum,* antibodies, cytokines, mice

Introduction

Gynostemma pentaphyllum (Cucurbitaceae) is a perennial herb plant that grows in wild fields of Southern China, Japan, Korea and Taiwan (Valentina *et al*.,2005). Common names for *G.pentaphyllum* include Jiaogulan in China and Taiwan, and Amachazuru in Japan (Lin *et al*.,2000). Since 1980, Chinese and Japanese scholars had studied and isolated gypenosides of more than 90 kinds (Valentina *et al*.,2005) from this plant. There were six gypenosides having the same ginesnoides structure as ginseng. (Kuwahark *et al*.,1989), so it also been called as the southern ginseng (Cui *et al*.,1999).

It was found that the extract of *G. pentaphyllum* has several pharmacological effects, including treatment for hyperlipidemia (Cour *et al*.,1995), cardiovascular disease (Circosta *et al*.,2005) and cancer (Lin *et al*.,1993). Besides, one of gypenosides from extract of *G. pentaphyllum* was found to have a bioactivity of anti-diabetic effect (Norberg *et al*.,2004). Thus, *G. pentaphyllum* acts like a kind of medical herbs with multiple clinic and basic research values.

Both T and B lymphocytes are important for having many functions in adaptive immunity (Szabo *et al*.,2003). Helper T (Th) cells are mainly divided into two subtypes, namely Th1 and Th2 cells (Abbas *et al*.,1996; Romagnani ,1997). Th1 cells can secret interleukin (IL)-2, IL-12, and interferon (IFN)-γ (Szabo *et al*.,2003); and Th2

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cells can produce IL-4, IL-5, and IL-13 (Glimcher and Murphy ,2000; McKenzie ,2000). In addition, B cells can secret Th1-associated cytokine induced IgG2a and Th2-associated cytokine induced IgG1 production (Szabo *et al*.,2003). Therefore, Th cells activities in turn are able to influence the pattern of IgG subtypes production by B cells (O'Garra and Arai ,2000).

This investigation was aim to reveal the immune responses particularly referred to cytokine and antibody production of oral administration of *G. pentaphyllum*. Previously, we had reported that *G. pentaphyllum* intraperitoneally injected mice could raise their IgG2a levels in serum and promote their Th1 and Th2-associated cytokines secretion from spleen cells cultured with Con A (Liou CJ *et al*., 2004). However, the traditional way of giving this medicine to a person is through the oral route. Thus, in this study, oral administrations as oppose to intraperitoneal injection of *G. pentaphyllum* were carried; and their immunological outcomes were compared using the same mouse model.

Materials and Methods

Preparation of G. pentaphyllum extracts

G. pentaphyllum extracts were prepared by the Department of Chinese Herbal Pharmacy, Chang Gung Memorial Hospital. Briefly, total dried plant (500g) of *G. pentaphyllum* was soaked in water and boiled for 50 minutes. The supernatant was filtered and then lyophilized. One hundred and eighty one grams of dry powder was obtained. The extract was reconstituted with phosphate buffered saline (PBS) before use. The solution was sterilized with 0.25 μm filters, and stored at -80 °C. The amount of GP extract (0.05 g, 0.5 g, and 5.0 g per Kg of body weight) given to each mouse was presented as the original weight of dried plant.

Animals and the administration of G. pentaphyllum extracts

Female BALB/c mice aged 6-8 weeks were purchased from the National Laboratory Animal Center, Taiwan. Mice were divided into four groups (11 mice per each group except 5G group 12 mice), and fed with normal saline (as the normal group, designated Normal) or various doses of *G. pentaphyllum* extracts, including 0.05 g, 0.5 g, and 5.0 g (total plant dry weight) per body weight daily (They were named 0.05G, 0.5G, 5G) for 5 consecutive days.

Serum collection and spleen cell cultures

Mice were sacrificed on day 6. Bloods were taken and centrifuged at 6,000 rpm for 20 min; and sera were collected and stocked at -80℃.

Single cell suspensions were prepared as previously described (Liou *et al.*, 2004). For antibodies assay, spleen cells ($5x10^6$ cells/ml) were cultured in a medium containing RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin/ streptomycin, and 1 μg/ml lipopolysaccharide (LPS) for five days. The cultured supernatants were collected from each day of incubation , and assayed their antibodies content with enzyme-linked immunosorbent assay (ELISA). To detect cytokine production, the spleen cells were cultured in presence of 1 μg /ml concanavalin A (Con A) for two days, and supernatants were assayed with ELISA technique.

ELISA assay

The sandwich ELISA technique was used to determine the concentrations of IgG1, and IgG2a. Microtiter plates were coated with 100 μl of rabbit anti-mouse anti-IgG1, or anti-IgG2a antibodies (R&D Systems, Minneapolis, MN, USA) in PBS. Following an overnight incubation at 4 °C, the plates were washed and blocked with 3 % BSA for 1 h at 37°C. After washing, 100 μl each of serial dilutions of standard IgG1, IgG2a, and dilutions of collected serum were added. Then, plates were incubated for 2 h at 37°C. Following that, secondary antibody (BD Biosciences, San Diego, CA, USA) was added and incubated for 2 h at 37°C. The plate was washed and added with conjugated-horseradish peroxidase (HRP). After that, plates were added with 100 μl of o-phenylenediamine (OPD) solution (0.4 mg/ml) for 20 min at room temperature. Finally, the reaction was stopped by adding 25 μl of 3N H_2SO_4. The optical density (OD) at 490 nm in each well was read using an ELISA reader. The limit of detection of the mouse IgG1 and IgG2a ELISA were 3.9ng/ml.

The amounts of IL-2, IFN-γ, IL-4 and IL-5 were measured using the ELISA kits specific for each cytokine (R&D Systems, Minneapolis, MN, USA). The minimum detectable concentration is 9.4 pg/ml for IFN-γ, 15.6 pg/ml for IL-2, 7.8 pg/ml for

IL-4, and 15.6 pg/ml for IL-5.

Histological examination

Liver and kidney were fixed and embedded in the OCT reagent, snapped-freeze in liquid nitrogen, and stored at -80°C. The tissue was sliced into 6 μm sections, and then stained with HE stain (contained hematoxylin and eosin) (Wu *et al.*, 2006).

Analysis ALT and AST of serum

Biochemical analyses of serum were used to detect enzymatic activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST). Owing to those biochemical assays need about 1ml serum, we had to pool several mice sera in order to obtain the meaningful biochemical results. In BALB/c mice, normal ranges for ALT and AST are 15-84 U/L and 54-298 U/L respectively (Alemany *et al.*, 2000; Mayer *et al.*, 2005).

Statistics analysis

Data were analyzed using the Student's *t*-test. Values were presented as the mean ± the standard error (SE). Probability values (*p*) of less than 0.05 were considered to be significant.

Results

Higher levels of immunoglobulin in mice serum were induced by G. pentaphyllum

Each group of BALB/c mice were fed with various doses (0, 0.05, 0.5, or 5 g/kg/day) of *G. pentaphyllum* for 5 consecutive days and then sacrificed on day 6. The mice were divided into 4 groups designated as Normal, 0.05G, 0.5G, and 5G groups, respectively. Serum antibody levels from sacrificed animals were examined at the end of experiments. Results showed that production of IgG1 in sera of *G. pentaphyllum* fed mice were all suppressed as compared with the Normal control (Fig. 1A, 1.51 ± 0.09 mg/ml for 0.05G, 1.25 ± 0.12 mg/ml for 0.5G, 1.04 ± 0.10 mg/ml for 5G *vs.* 1.97 ± 0.12 mg/ml for the Normal, $p<0.05$, $p<0.01$ or $p<0.01$, respectively). On the other hand, *G. pentaphyllum*-treated groups exhibited a dose-dependent enhancement on their serum IgG2a production, but only the 5G group appeared significantly higher when compared to the Normal (Fig. 1B, 1.12 ± 0.13 mg/ml for 0.05G, 1.50 ± 0.18 mg/ml for 0.5G, and 1.87 ± 0.14 mg/ml for 5G *vs.* 1.17 ± 0.14 mg/ml for the Normal, $p=0.79$, $p=0.14$, or $p=0.007$, respectively).

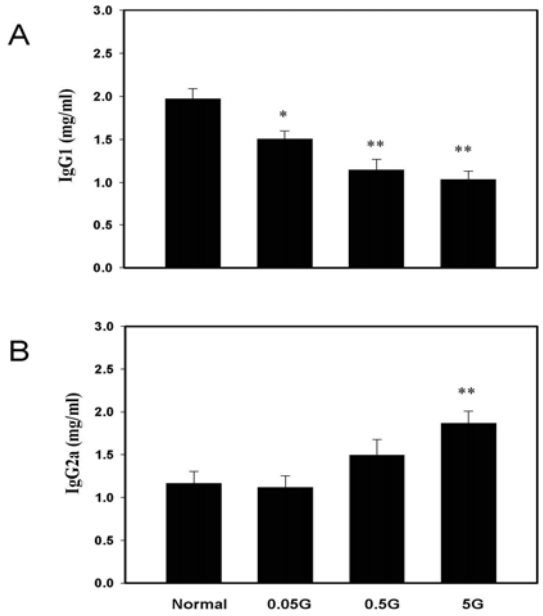

Figure 1. *G. pentaphyllum* extracts induced antibodies secretion in serum. Sera were collected from mice after oral administration of *Gynostemma pentaphyllum* extracts for 5 consecutive days (each group n=11 expect for 5G group n=12). The mice were designated as Normal, 0.05G, 0.50G, and 5.00G groups for various doses (0, 0.05, 0.50, or 5.00 g/kg/day) of *G. pentaphyllum* extract given respectively. The concentrations of serum IgG1 (**A**), and IgG2 (**B**) were determined with ELISA. Data were presented as mean ± standard error (SE). * indicates $p<0.05$, and ** indicates $p<0.01$ compared to the serum level of Normal group.

G. pentaphyllum enhanced immunoglobulin production from spleen cells

The supernatants of spleen cells culture were collected after stimulation with 1 μg/ml of LPS for 5 days. Antibody levels from supernatants were calculated from ELISA data. A dose-dependent decrease of IgG1 secretion vs. increased concentrations of *G. pentaphyllum* treatment (Fig. 2A) was seen. Compared to the Normal group, the IgG1 production was suppressed about 2 folds from 0.05G spleen cells, and 3 folds from 0.5G and 5G spleen cells (0.100 ± 0.013 μg/ml for 0.05G, 0.073 ± 0.012 μg/ml for 0.5G, and 0.071 ± 0.009 μg/ml for 5G *vs.* 0.214 ± 0.019 μg/ml for the Normal, $p<0.01$, $p<0.01$, or $p<0.01$, respectively). Whereas results in Fig. 2B indicated that activated spleen cells from the highest dose of 5G group had a more prominently enhanced IgG2a secretion than the

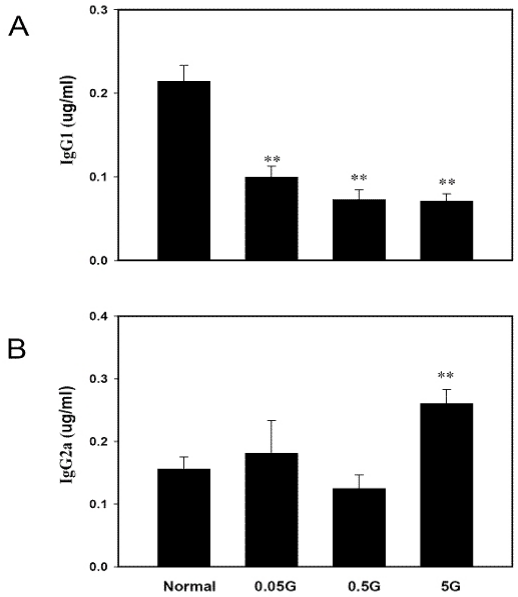

Figure 2. *G. pentaphyllum* promoted immunoglobulin production from activated spleen cells. Spleen cells ($5x10^6$ cells/ml) were incubated with 1 μg/ml of LPS for 5 days. Supernatants were collected and detected with ELISA. The concentrations of IgG1 (**A**) and IgG2a (**B**) were measured. Data were presented as mean ± SE. * indicates $p<0.05$, and ** indicates $p<0.01$, compared to the Normal group.

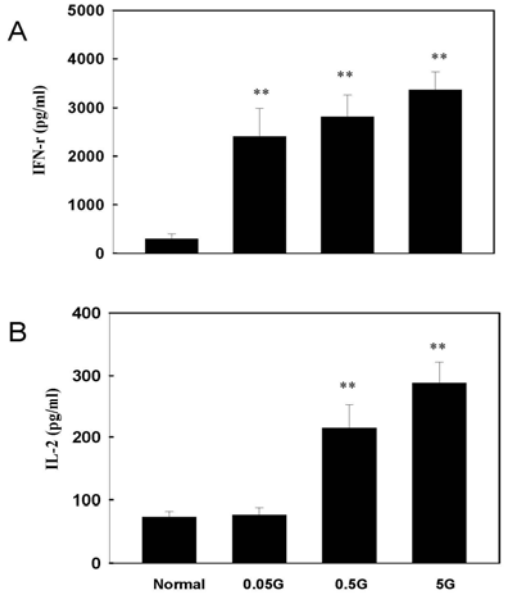

Figure 3. *G. pentaphyllum* enhanced production of Th1-associated cytokines from activated spleen cells. Spleen cells ($5x10^6$ cells/ml) were incubated with 1 μg/ml of Con A for 2 days. Culture supernatants were collected, and their concentrations of IFN-γ (**A**), IL-2 (**B**) were measured by ELISA. Data were presented as mean ± SE. * indicates $p<0.05$, and ** indicates $p<0.01$, compared to the Normal group.

Normal (0.26 ± 0.02 μg/ml for 5G *vs.* 0.16 ± 0.02 μg/ml for the Normal, $p<0.01$, respectively).

G. pentaphyllum modulated the production of cytokines from activated spleen cells

Spleen cells of 5 x 10^6 cells/ml were cultured with Con A for 2 days. Fig. 3A showed the more *G. pentaphyllum* extracts taken, the more enhanced Th1-type cytokine, IFN-γ, production from Con A-activated spleen cells (compared to 295.6 ± 100.3 pg/ml for the Normal group, 2408.8 ± 573.5 pg/ml for 0.05G, $p<0.01$; 2812.8 ± 436.7 pg/ml for 0.5G, $p<0.01$ or 3357.7 ± 369.2 pg/ml for 5G, $p<0.01$). Likewise, IL-2 was another Th1-type cytokine that showed a dose-dependent increase response (Fig. 3B, 73.4 ± 8.9 pg/ml for the Normal group, 76.6 ± 11.9 pg/ml for 0.05G, $p=0.77$; 214.4 ± 36.9 pg/ml for 0.5G, $p<0.01$; and 288.2 ± 33.3 pg/ml for 5G, $p<0.01$).

Th2-type cytokines including IL-4 and IL-5 were also chosen for comparison. We had found IL-4 secretions from cultured spleen cells was the highest in 0.05G group, whereas 0.5G and 5G groups were found to be lower than the Normal group (Fig. 4A, 137.3 ± 11.6 pg/ml for the Normal group, 466.52 ± 49.4 pg/ml for 0.05G, $p<0.05$; 93.2 ± 6.1 pg/ml for 0.5G, $p<0.05$; and 86.5 ± 5.9 pg/ml for 5G, $p<0.01$). On the other hand, the IL-5 production was not significantly decreased or increased in mice fed with various dosages of *G. pentaphyllum* (Fig. 4B, 157.3 ± 26.3 pg/ml for the Normal group, 193.5 ± 35.2 pg/ml for 0.05G, $p=0.317$; 168.3 ± 33.7 pg/ml for 0.50G, $p=0.715$; and 147.3 ± 29.7 pg/ml for 5.00G, $p=0.793$).

Effect of G. pentaphyllum extract on blood biochemistry and histopathology of internal organs

We also wanted to know whether a short-term oral *G. pentaphyllum* regimen might exhibit certain toxic effects on internal vital organs such as liver or kidney. For histopathological examination, normal and drug-treated liver and kidney tissues were thin sectioned, histochemical stained, and examined under microscope to detect any abnormalities (Fig. 5). Results of histopathological examination of liver and kidney showed that morphology of cells between every group of mouse had no apparent differences from each other. Regarded to liver functions, serum levels of ALT and AST were found that were still within the normal reference

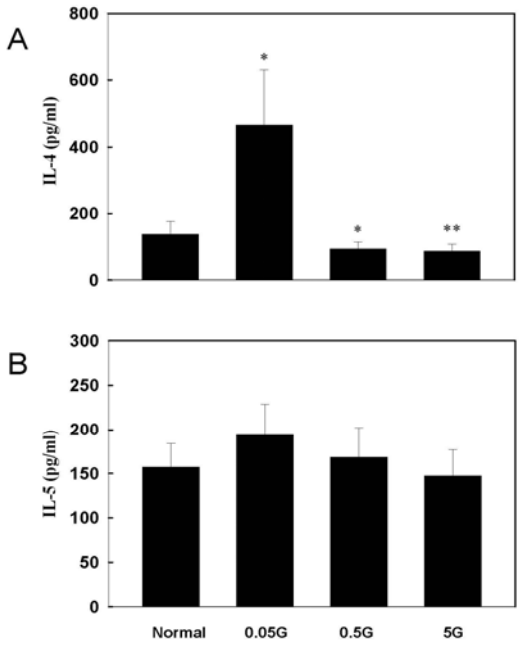

Figure 4. Inability of Th2-associated cytokines production of from activated spleen cells by *G. pentaphyllum* given orally. Spleen cells ($5x10^6$ cells/ml) were incubated with 1 μg/ml of Con A for 2 days. Culture supernatants were collected and the concentration of IL-4 (**A**) and IL-5 (**B**) were measured by ELISA. Date was presented as mean ± SE. * indicates $p<0.05$, and ** indicates $p<0.01$, compared to the Normal group.

Table 1. Biochemical values of Balb/c mice receiving G. pentaphyllum, including ALT and AST of serum (n=2).

Group	ALT	AST
N	42.0±0.0	93.5±2.5
0.05G	80.0±13.0	105.0±4.0
0.5G	77.5±11.5	126.0±10.0
5G	59.5±15.5	103.5±1.5

range indicating no significant increases in *G. pentaphyllum* extract-treated groups (Table 1). All these results showed that the *G. pentaphyllum* extract-treated mice did not produce any detectable dose-related serum biochemical and histopathological changes or damages in their liver and kidney.

Discussion

enhancing Th1 and Th2-associated cytokines secretion by spleen cells (data no shown). However, oral intake an extract of traditional Chinese medicine is the most common practice in China for thousands of years rather than taking through the intraperitoneal injection route. In this regard, we have undertaken the investigation trying to understand immunological outcomes by the oral *G. pentaphyllum* administration as oppose to intraperitoneal intake method.

Sera and LPS stimulated culture spleen cells were collected and analyzed for their antibody productions. It was found that *G. pentaphyllum* could suppress IgG1 secretion and promote IgG2a levels. Since IgG1 is belonged to Th1-associated antibody and IgG2a is belonged to Th2-associated antibody (O'Garra and Arai, 2000), it is evidently that oral *G. pentaphyllum* taking can regulate the IgG subtype production in mice. As various Th-type cells have the ability to influence IgG1 and IgG2a secretions through their cytokines production (O'Garra and Arai, 2000), so cultured spleen cells from the same animals were stimulated with Con A and checked for cytokines secretion. Th1 cells associated cytokines of IL-2 and IFN-γ and Th2

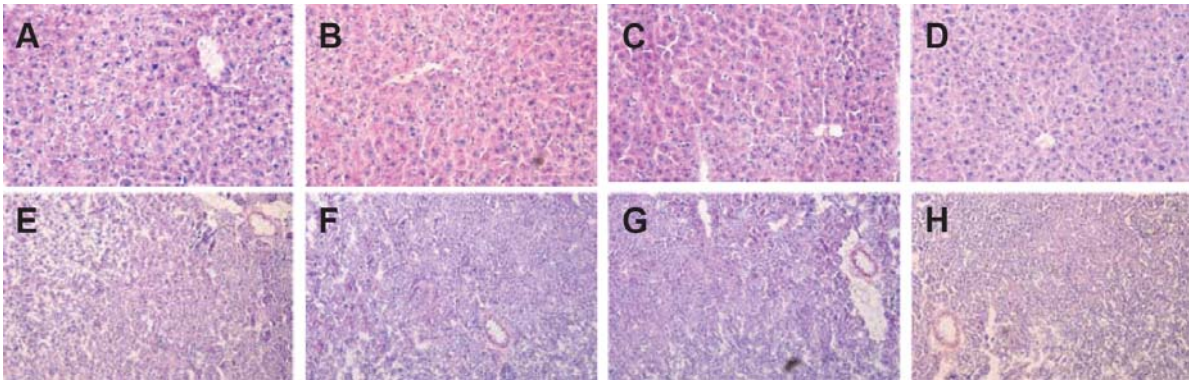

Figure 5. HE stain of liver and kidney slices from each group. Sections were magnified to x200. From (**A**) to (**D**) were liver sections of Normal, 0.05G, 0.5G, and 5G group, respectively. From (**E**) to (**H**) were kidney sections of Normal, 0.05G, 0.5G, and 5G group, respectively.

cells associated cytokines of IL-5 and IL-4 were assayed (Romagnani, 1997). Our results found that mice fed with more *G. pentaphyllum* resuled in more Th1 cell associated cytokines IL-2 and IFN-γ secretions. Because IFN-γ can induce IgM antibody class-switch to IgG2a in B cells (Abbas *et al*., 1996), thus our data may suggest that orally taking of *G. pentaphyllum* can lead spleen cells to secret more IFN-γ; and in-turn, to induce more B cell to secret IgG2a. IL-2 is a T cell growth factor that can stimulate and enhance T cell proliferation (Smith, 1988). It can also promote the general immunoglobulin synthesis and J-chain transcription in B cells (Johansen and Brandtzaeg, 2004). Regarded to Th2 cell representing cytokines, our results showed that amounts of IL-5 secretion between Normal group and all other orally *G. pentaphyllum* taking groups were similar, in contrast to 0.5G and 5G groups of another Th2 cell representing cytokine IL-4 which were produced in lesser amounts than the Normal group. It has been suggested that the function IL-4 is more important in induction of IgM class-switch to IgG1 (Glimcher and Murphy, 2000). Therefore, decreased IL-4 secretion can lead to reduced IgG1 production after oral intake of *G. pentaphyllum*.

It was not a worrisome that IL-5 secretions from spleen cells had not significantly increased or decreased after oral *G. pentaphyllum* regimen. Previous papers reported that oral intake of Fu-Ling could promote IL-4 secretion (did not assay for IL-5), but oral intake of Ginseng extract could not promote or decrease IL-4 production (Liou *et al*., 2002; Liou *et al*., 2005). Considered the homeostatic maintenance of normal physiological status in normal mice, any drug given orally change their immunity in every aspect drastically may not proper.

G. pentaphyllum is usually taken as a health-tea in China and Taiwan. Besides, reports indicated that *G. pentaphyllum* is able to decrease the incidence of hyperlipidemia in which lipid deposit in the artery wall and the artery sclerosis occurred (Cour *et al*., 1995; Megalli *et al*., 2005), and to inhibit growth of hepatoma cells. (Wang *et al*., 2002). However, the effective compounds with immune modulation function have not been reported. In this study, we provide the first evidence to demonstrate the orally administration of 5g/kg of oral *G. pentaphyllum* regimen can enhance serum immunoglobulin levels. Our results could enhance non-specific Th1 and suppress Th2 immune response. We will apply a murine allergic model to assay whether *G. pentaphyllum* can decrease Th2 and airway inflammatory responses. However, whether oral *G. pentaphyllum* extracts could cause higher Th1 responses and then generate unexpected side effects require further studies.

We had worried about this dosage was too high. However, a 750mg/kg of *G. pentaphyllum* was reported given orally to Wistar rat up to 24 continuous weeks by others (Attawish *et al*., 2004); and there was no toxicity detected using any relevant tests in the same experiment. Thus, no toxic effects manifested in their long-term oral *G. pentaphyllum* regimen. Though our experiment lasted only five days, it was apparently safe for us to give the highest dosage of 5g/kg orally. If there was toxicity existed, it should be very moderate. We had measured ALT and AST for liver function, and found that even though the oral *G. pentaphyllum* intake mice had higher enzymatic activities than the Normal group; however, it were not too high exceeding the normal reference range (Mayer *et al.*, 2005; Alemany *et al*., 2000). No abnormal changes were found in histochemically stained sections of liver and kidney tissues which also indicated that it is safe to take *G. pentaphyllum* orally.

In conclusion, our results suggest that oral administration of *G pentaphyllum* extracts could improve Th1 associated immune response. Besides, there were no any acute toxic effects as indicated from serum ALT and AST levels and histopathological section changes of liver and kidney tissues in *G. pentaphyllum* treated mice.

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小鼠短期口服絞股藍萃取液可以增進 Th1 細胞的活性

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摘　要

絞股藍 (*Gynostemma pentaphyllum*) 廣泛的分佈在中國南部、南韓與日本，在中國與有些亞洲國家，絞股藍被拿來當作保健茶飲用。我們曾嘗試將絞股藍的萃取液，連續五天腹腔注射到小鼠的腹腔，發現可以提升血液 IgG2a 的增加，脾臟細胞用 Con A 刺激後培養兩天，也可以發現 Th1 及 Th2 細胞相關的細胞激素會有上昇的現象。本實驗我們使用正常給藥的口服方式，觀察免疫反應表現是否與腹腔注射給藥一樣。實驗結果證明，短期口服絞股藍不僅可以增進血液 IgG2a 的量，也會促進脾臟細胞培養後 Th1 細胞相關的細胞激素增加，並且抑制 Th2 細胞相關的細胞激素 IL-4 的分泌量。

關鍵詞：絞股藍、抗體、細胞激素、小鼠

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