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# Immunosuppressive Effect of the Intraperitonially Injected Pine Needle Distillate in Mice

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## Abstract

**This study examined the effect of pine needle distillate (*Pinus densiflora* Sieb. et Zucc) on the immune system and hematological parameters. C57BL/6 male mice weighing 20~21 g were divided into 3 groups and intraperitonially injected with either 200 μL of saline (control), 50% diluted (P50) or 100% pine needle distillate (P100) once a day for 24 days. At the end of the experiment, the mice were anesthetized by ether and peripheral blood was collected from the femoral artery and the spleen was excised. Spleen weight decreased significantly ($p<0.001$) in the pine needle groups compared to the control group. The blood was used for a complete blood count and flow cytometrical analysis after immunofluorescence staining. The pine needle distillate dose-dependently decreased the $CD4^+/CD8^+$ ratio ($p<0.05$), and showed a tendency to increase the mean FSC (forward scatter) values of the $CD8^+$ T cells, while decrease the values of the $CD4^+$ T cells. There were no significant differences in WBC, RBC and platelet counts among the three groups, but hemoglobin and hemoglobin-related parameters and platelet volume increased and red blood cell volumes decreased with the administration of the pine needle distillate. These results suggest that the pine needle distillate may have immunosuppressive effects.**

**Key words:** immunosuppression, CD4, CD8, T cell, complete blood count, mouse, pine needle distillate

## INTRODUCTION

The pine tree (*Pinus densiflora* Sieb. et Zucc) is sometimes referred as a miracle medicinal plant, and has long been used as a folk remedy and health food. Pine needles are used in folk medicine to treat liver diseases, gastrointestinal diseases, nervous system diseases, circulatory diseases, and skin problems (1,2). Numerous studies have investigated the physiological functions of pine needles. One study reported that dried pine needle powder and ethanol extracts of pine needles decreased total lipids, triglycerides and cholesterol concentrations, and had antioxidative properties in rats (3). Another study found that supplementing rats with pine needle powder improved liver function through the activation of sALT, sAST, and SOD (4). When rats are fed with a high cholesterol diet, pine needle powder can reduce serum total cholesterol levels and liver TBARS (5). Ethanol extracts of pine needles have also shown to act synergistically with an anti-tumor substances in enhancing the anti-tumor effects (6); *in vitro* (6,7) and *in vivo* experiments (6) have shown that pine needle extracts can suppress tumor growth or act as antitumor agents. Furthermore, it was found that the ethanol extracts of pine needles suppress mutagenicity (8,9), and exhibit antibiotic properties (10). We previously demonstrated that pine needle distillate exerts *in vitro* cytotoxicity against several cancer cell-lines including breast cancer, leukemia and liver cancer (11). To date, pine needle extracts have been found to lower blood lipid levels, and to exert antioxidative, antitumor and antimutagenic effects (3-9); however, few studies have evaluated the effects of pine needles and their extracts on the immune system.

The major cellular components of the immune system are T cells, B cells, NK cells, monocytes, and macrophages. Many of the activities of immune cells are carried out by chemical molecules and proteins such as antibodies, complement, and various cytokines. Lymphocytes such as T cells and B cells are well-known to have immunological memories. T cells help B cells in producing antibodies, but also play a major role in cell-mediated immunity. Even though these T cells do not have distinctive morphological features distinguishing them from other lymphocytes, they can be distinguished due to their characteristic molecules or markers on their cell surfaces. The representative surface markers of T cells are CD3, CD4 and CD8 molecules. All T cells have CD3 molecules and T cells in the

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peripheral blood also have either CD4 or CD8 molecules. Thus T cells can be subdivided into two functionally distinguished subtypes; $CD4^+$ T cells and $CD8^+$ T cells. T cells expressing CD4 molecules are helper/inducer cells, and T cells with CD8 molecules are suppressor/cytotoxic cells (12-14).

Although the immune system protects the body from endogenous and exogenous pathogens, improper immune responses such as autoimmune reactions or transplant rejections can be life-threatening. The effective use of immunosuppressants is a major factor in determining the success of organ transplantations. Immunosuppressive reactions limit specific immune responses to external antigens and render the body's own antigens unresponsive, thus preventing harmful reactions in activated lymph nodes (15).

Accordingly, immunosuppressants have been developed to minimize rejection reactions; non-specific agents against Tc (cytotoxic T) cells or $T_H$ (helper T) cells such as steroids, anti-nucleic acid, anti-transcription factors, and specific agents for T cells such as anti-lymphocyte antibodies, are well known. However, the long-term use of these agents may cause negative effects such as hyperlipidemia, hypertension, diabetes, reduction of platelets and white blood cells, renal inflammation due to the development of antigen-antibody complexes, and fever (16). In addition, new immunosuppressants, such as suppressants of cytokine synthesis, cytokine growth factor, DNA synthesis, and cell maturity, have improved the treatment of rejection reactions. However, these newer drugs still have serious effects such as renal toxicity, infection due to excessive suppression of immunity and increased risks of cancer. Therfore, much efforts have been devoted to the search for natural immunosuppressive substances which do not have harmful side effects.

This study evaluated the immunosuppressive and/or immunoactive effects of distilled pine needle extracts. We reports here that pine needle distillate exerted immunosuppressive, rather than immunoactive, effects.

## MATERIALS AND METHODS

### Experimental animals

Six week old, male C57BL/6 mice, weighing 20~21 g, were obtained from the Korea Research Institute of Chemical Technology. After 10 days of acclimatization in the laboratory, animals were assigned to experimental groups and to a control group using a randomized complete block design. The laboratory was maintained at a constant temperature of $23\pm2^{\circ}C$, relative humidity of $55\pm5\%$, and a 12/12 h light and dark cycle. Animals were given free access to commercial feed (Sam-Yang Ration Co., Korea) and filtered tap water.

### Manufacture of distilled pine needle extracts

Pine needles (*Pinus densiflora* Sieb. et Zucc) were collected, washed with clean water, and dried. The pine needles were squeezed under high pressure, and the resulting liquid distilled. The distilled pine needle extract was used for peritoneal injection into the experimental animals.

### Experimental groups and diets

Experimental animals were divided into three groups. Each group was given a daily intraperitoneal injection of 200 μL of saline or pine needle distillate for 24 days, as follows: the control group, saline; the low dose experimental group, 50% pine needle distillate (P50); and the high dose experimental group, 100% pine needle distillate (P100).

### Blood collection and spleen excision

At the end of the experiment, food was withdrawn for 24 hours after which the mice were weighed and then anaesthetized with ethyl ether. Blood was collected in heparinized micropipettes from the femoral artery and then the animals were sacrificed by cervical dislocation, and the spleen was excised. Twenty μL of the blood was used for the flow cytometrical analysis after immunofluorescence staining, as described later. The rest of the blood was used for complete blood counts (CBC) using an automatic analyzer. The spleen was weighed after removal of the fat tissues.

### Immunoflorescence staining and flow cytometrical analysis

Flow cytometrical analysis was carried out to investigate the effects of distilled pine needle extracts on $CD4^+$ and $CD8^+$ T cells. When the immunofluorescent stained cells pass through a narrow quartz flow cell of a flow cytometer, they reflect the precisely focused laser beam and emit fluorecence. By detecting and analyzing the deflections of the laser beam itself and the fluorescence, not only the size of the cells but also the antigen profile and other various characteristics of the cells can be determined. For example, the quantity of the forward scattered light (FSC) at a $3\sim9^{\circ}$ degree angle and side scattered light (SSC) at about a $90^{\circ}$ degree angle, respectively, matches well with the relative size and the granularity of the cells. When lymphocytes are activated, enlarged lymphoblasts, which exhibit higher FSC values, are formed; and then cell division occurs. Therefore, a high FSC score implies activation of immune cells. In this experiment, we used one-step two color immunofluorescence (IF) staining with phycoerythrin (PE)-conjugated anti-mouse CD8 monoclonal antibody (mAb) and

fluoreseinisothiocyanate (FITC)-conjugated anti-mouse CD4 mAb to label $CD4^+$ and $CD8^+$ T cells, respectively. In short, 20 μL of the blood was mixed with 10 μL of the diluted (1:10) PE-conjugated anti-mouse CD8 mAb and 10 μL of FITC-conjugated anti-mouse CD4 mAb, and this mixture was then incubated on melting ice for 30 minutes. After incubation, the red blood cells were hypotonically lysed with 0.75 mL of 0.83% ammonium chloride (in 0.01 M Tris-HCl buffer, pH 7.5 ± 0.2). After washing twice with PBS, the cells were suspended in 500 μL PBS. Three to five minutes prior to analysis, 50 μL of propidium iodide (PI) solution (250 μg/mL) was added to distinguish dead cells and then the cells were analyzed by using a FACScalibur flowcytometer (Beckton Dickinson Co.) as described elsewhere (17,18).

### Blood analysis

RBC, WBC, platelets, hemoglobin concentration (HB), and RBC indices (19) were analyzed by using the ADVIA 120 blood autoanalyzer (Bayer Co.)

### Statistical analysis

Data were expressed as mean values ± standard deviations. Differences among the means of the individual groups were assessed by one-way ANOVA with Duncan's multiple range test (SPSS 10.0, SPSS Institute, USA). Differences of $p < 0.05$ were considered significant (20).

## RESULTS AND DISCUSSION

### Body and spleen weight

Final body weights were similar among all groups; however, relative spleen weights were 60.2% lower in the high dose group, treated with 100% pine needle distillate (the P100 group), and 51.5% lower in the low dose experimental group, treated with diluted pine needle distillate (the P50 group), compared to the control group ($p < 0.01$) (Table 1). Spleen weight typically increases when the immune system is activated, especially when a large quantity of antigen is introduced into the body, but decreases when bone marrow function is suppressed or when there is a die-off of immune cells (21). Therefore, the marked decrease in spleen weight (over 50%) strongly implies that the pine needle distillates exerted immunosuppressive effects. However, further research is needed to ascertain whether the reduced spleen weights can be attributed to the suppression of bone marrow or to increased destruction of white blood cells.

### Effects on $CD4^+$ and $CD8^+$ T cells

In general, $CD4^+$ T cells function as $T_H$ cells by secreting a variety of cytokines and by activating B cells, Tc cells, monocytes and macrophages, while $CD8^+$ cells are cytotoxic Tc cells or immunosuppressive Ts cells (15); thus, the activation of $CD4^+$ generally implies an overall activation of the immune system (10,15). After the mice were injected intraperitoneally with pine needle distillate, the FSC values of $CD4^+$ T cells were decreased by 1.11 ~ 2.26%, while those of $CD8^+$ T cells increased by 0.9 ~ 1.54%, although the differences were not statistically significant (Table 2). These results suggest that immunosuppression was occurring from the supression of $CD4^+$ T cell and activation of $CD8^+$ T cell, which can act as supressor T cells. The statistical insignificance, however, could be attributed to the lapse of 24 days which may be too long to measure changes in FSC values due to lymphoblast production early in the immune response. However, as illustrated in Fig. 1, the $CD4^+$/ $CD8^+$ ratio was dose-dependently lower in both the P50 and P100 groups ($p < 0.05$) compared to the control, suggesting that pine needle distillates increase the number of $CD8^+$ T cells which have immunosuppressive functions, while simultaneously decreasing $CD4^+$ T cells, thereby reducing the $CD4^+/CD8^+$ ratio. The significant change in the $CD4^+$/ $CD8^+$ ratio is in contrast to the insignificant change in FSC values, which may be because the experimental period of 24 days was long enough for the larger lymphoblasts, which have higher FSC values, to continue the subsequent proliferation into functionally active lymphocytes, which have lower FSC values.

### Hematology of WBC, RBC, and platelets

There were no significant differences in WBCs, RBCs, and platelet counts among these groups; however, hemoglobin and hemoglobin-related parameters, and platelet volume increased and red blood cell volumes decreased

**Table 1.** Effect of pine needle distillate on mice spleen weight

| Group | n[1] | Body weight (g) | Spleen weight (mg) | Spleen weight (mg)/ Body weight (g) |
|---|---|---|---|---|
| Control | 6 | 24.2 + 0.9[2)NS3)] | 144.1 ± 51.4[b4)] | 5.98 ± 2.15[b4)] |
| P50[5)] | 6 | 23.4 + 1.1 | 63.0 ± 15.7[a] | 2.69 ± 0.68[a] |
| P100[6)] | 6 | 23.7 + 1.0 | 52.1 ± 6.1[a] | 2.21 ± 0.35[a] |

[1)]Number of mouse used.
[2)]Mean ± SD.
[3)]NS: not significant.
[4)]Values within a column with different superscripts are significantly different at $p < 0.05$ by Duncan's multiple range test.
[5)]P50: 50% dilutiled pine needle distillate. [6)]P100: 100% pine needle distillate.

**Table 2.** Effect of pine needle distillate on $CD4^+$ and $CD8^+$

| Group | | $CD4^+$ | | | $CD8^+$ | | |
|---|---|---|---|---|---|---|---|
| | | Event | FSC | | Event | FSC | |
| | | | Mean value | % increase[3] | | Mean value | % increase |
| Control | 6 | 993±541[1)NS2)] | 240.6±3.9[NS] | 0 | 391±188[NS] | 253.6± 5.5[NS] | 0 |
| P50[4] | | 1017±605 | 235.1±3.5 | -2.26 | 506±152 | 255.8± 9.9 | 0.90 |
| P100[5] | 6 | 564±587 | 237.9±7.2 | -1.11 | 387±214 | 257.5±10.3 | 1.54 |

[1]Mean±SD.
[2]NS: not significant.
[3]% increase=100×(T-C)/C, where T is the values of mean FSC of the treated group, C is the values of mean FSC of the control.
[4]P50: 50% dilutiled pine needle distillate. [5]P100: 100% pine needle distillates.

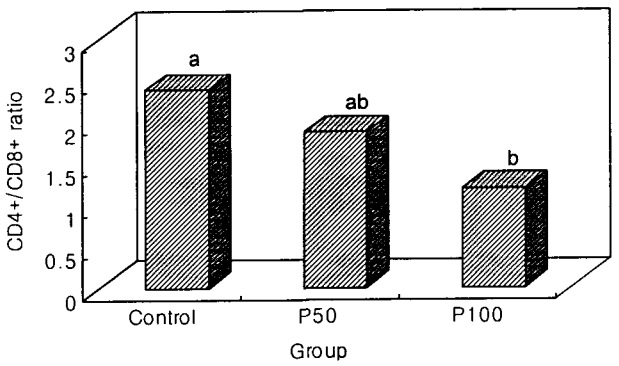


**Fig. 1.** Effect of pine needle distillate on the $CD4^+/CD8^+$ ratio. All values are mean ± SD. Bars with different letters are significantly different at $p<0.05$ by by Duncan's multiple range test. P50: 50% dilutiled pine needle distillate, P100: 100% pine needle distillate.

in the groups administered the pine needle distillates. (Table 3). Although not statistically significant, the numbers of platelets were slightly lowered, dose-dependently, in the pine needle treated groups ($328\times10^3/mm^3$ in the P100 group and $410\times10^3/mm^3$ in the P50 group) compared to the control group ($433\times10^3/mm^3$). On the other hand, mean platelet volume (MPV) and platelet distribution width (PDW) were highest in the P100 group, followed by the P50 group. Mean corpuscular volume (MCV) is a measure of the average size of a single RBC, and it is derived by dividing hematocrit by the total RBC count. As the concentration of pine needle extract increased, MCV decreased and was significant in the P100 group ($p<0.001$). MCV values are known to increase in the case of vitamin $B_{12}$ deficiency, folic acid deficiency, chronic liver diseases, alcoholism, and chemotherapy (22). Among hemoglobin indices, MCH (Mean Corpuscular Hemoglobin = hemoglobin/RBC count: a measure of the average amount of hemoglobin within an RBC) and MCHC (mean corpuscular hemoglobin concentration = hemoglobin × 100/hematocrit : a measure of the average concentration of hemoglobin within a single RBC) values were significantly higher in the P100 group ($p<0.001$), and also increased in a dose-dependent manner.

**Table 3.** Hematological values of mice administered with pine needle distillate

| Items[6] | Control | P50[4] | P100[5] |
|---|---|---|---|
| WBC ($\times10^3/mm^3$) | 3.8±1.8[1)NS2)] | 4.4±1.6 | 3.1±1.9 |
| Platelet count ($\times10^3/mm^3$) | 433.0±137.7[NS] | 410.2±24.9 | 328.8±25.2 |
| RBC ($\times10^6/mm^3$) | 8.7±0.7[NS] | 9.3±0.2 | 9.1±0.3 |
| Hb (g/dL) | 12.9±0.8[a3)] | 14.3±0.2[b] | 14.2±0.4[b] |
| Hct (%) | 43.5±5.2[ab] | 47.1±3.6[b] | 39.4±3.0[a] |
| MCV (fl) | 50.3±2.8[b] | 50.5±3.3[b] | 43.2±2.6[a] |
| MCH ($\rho$ g) | 15.0±0.4[a] | 15.3±0.2[ab] | 15.6±0.3[b] |
| MCHC (g/dL) | 29.9±2.4[a] | 30.5±2.2[a] | 36.1±2.3[b] |
| RDW (%) | 20.5±4.6[NS] | 20.2±3.6 | 22.5±2.0 |
| MPV (fl) | 9.4±5.2[a] | 10.7±5.1[a] | 16.7±1.9[b] |
| PDW (fl) | 9.1±1.9[a] | 9.5±1.9[a] | 12.7±2.3[b] |

[1]Mean±SD.
[2]NS: not significant.
[3]Values those in the same row with different superscripts are significantly different at $p<0.05$ by Duncan's multiple range test.
[4]P50: 50% dilutiled pine needle distillate. [5]P100: 100% pine needle distillate.
[6]WBC(white blood cells), RBC (red blood cells), Hb (hemoglobin), Hct (hematocrit), MCV (mean corpuscular volume), MCH (mean corpuscular hemoglobin), MCHC (mean corpuscular hemoglobin concentration), RDW(red cell distribution width), MPV (mean platelet volume), PDW (platelet distribution width).

Pine needles not only contain many nutrients such as ascorbic acid (29 mg/100 g), carotene (3100 μg/100 g), vitamin B (about 1 mg/100 g), as well as carbohydrates, protein, fat, and dietary fiber (23), but also contain non-nutrient bioactive compounds: bitter taste-ingredients, many kinds of volatile compounds including terpenoids or essential oils such as monoterpenes, limonene, β-pinene, α-pinene, and camphene, etc., most of which are water-insoluble (24,25), and also water soluble compounds including organic acids and their esters such as benzoic and cinnamic acids (26,27), coumaric acid esters (28), polysaccharides (29), polyphenol (30) or flavonoids such as quercetin and kaempferol and flavonoid glycosides (31). Although the active compound responsible for immunosuppressive effects can not be inferred from this study, it can be assumed that they are water-soluble and water-insoluble compounds with low molecular weights. Further research on immunologic properties of pine needles and characterization of the active compounds is needed.

In summary, this study found that pine needle distillates 1) significantly decreased spleen weights, 2) decreased the ratio of $CD4^+$ T cells ($T_H$ cell) to $CD8^+$ T cells, and 3) increased the FSC values of $CD8^+$ cells which are immunosuppressive. These results suggest that pine needle distillate has immunosuppressive properties; thus raising the possibility of using pine needle distillate for decreasing tissue and organ transplant rejection and for improving the longevity of the patients.

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