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Oriental Pharmacy and Experimental Medicine 2005 **5(2)**, 100-109

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Antioxidant activity and anti-tumor immunity by *Propolis* in mice

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SUMMARY

In South America, natural products with unknown drug effects are used as folk remedies and for preventive medicine. Among South American natural products, we directed our attention to *Propolis*, which have been known as medicinal plants, and examined the mechanisms by which these substances affect antioxidant activity, anti-tumor activity and immunoresponse. When the antioxidant activities of *Propolis* were examined by the DPPH and Rhoudan iron methods, since *Propolis* contains high levels of flavonoids, it is thought that flavonoids may be responsible for the antioxidant activity in this study. In the examination of immunoenhancement activity, we measured lymphocyte versus polymorphonuclear leukocyte ratios (L/P activity). The number of lymphocytes was significantly increased in groups treated with *Proplolis*. Specifically, slightly high levels of IFN-γ were measured in mice bearing the S-180 carcinoma, after administration of *Propolis*. This strongly suggests that cellular immunity is especially activated by treatment with *Propolis*, because production of IFN-α is limited to the T cells and NK cells stimulated by mitogen and sensitized antigen. TNF-α shows a different extent and mechanism of action depending on the target cells. When TNF-α was measured in mice bearing the S-180 carcinoma, mice treated with *Propolis* showed slightly higher TNF-α levels as compared to the control group. This suggests that activated macrophages produce TNF-α in mice treated with *Propolis*, since activated macrophages and lymphocytes are the source of most TNF-α. When anti-tumor action was examined using two kinds of sarcoma (Ehrlich solid carcinoma and Sarcoma-180 carcinoma), tumor-suppressive ratios after treatment with *Propolis* was 29.1%. When Sarcoma-180 solid carcinoma was used, tumor-suppressive ratios were 62%. Thus, *Propolis* showed strong anti-tumor activity against two kinds of solid carcinoma. Taken altogether, this strongly suggests that *Propolis* enhances original functions of macrophages and NK cells, and as a result, secondarily enhances the immune reaction and suppresses tumor growth.

Key words: *Propolis*; Immunoehhancement activity;.Antitumor activity ·

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INTRODUCTION

Individual differences are found in the susceptibility

to cancer, and prevention of carcinogenesis is possible (Sapi *et al.*, 2004). In South America, natural products with unknown drug effects are used as folk remedies and for preventive medicine. Among South American natural products, we directed our attention to *Propolis*, which have been known as medicinal plants, and examined the mechanisms by which these substances affect antioxidant activity (Usami *et al.*, 2004), anti-tumor activity (Leitao *et al.*, 2004) and immunoresponse (Matsui *et al.*, 2004).

Propolis is a resin prepared by combining leaf bud and sap collected by honey bees with secreted salivary juice, bee solder and pollen. The taste and smell of *Propolis* differs depending on the species of tree. Therefore, ingredients are subtly different. Honeybees combine sap with saliva, and then used it for repairing beehives. This keeps the interior of the beehives warm and sterile. Major constituents of *Propolis*, a natural antibiotic, are flavonoids, organic acids, phenols, various kinds of enzymes, vitamins and minerals (Usami *et al.*, 2004). Flavonoids are further classified into about 2000 species such as flavone, flavonol, and flavanone. Flavonoids promote the formation and regeneration of cells and tissues which are resistant to the invasion of viruses and bacteria by promoting cell activation involved in clearing of the blood and strengthening of the cellular membranes. Thus, *Propolis* has many physiological functions, and there are reports that *Propolis* has important direct actions on capillary vessels and in reducing inflammation (Leitao *et al.*, 2004). These activities include antibacterial action (Matsui *et al.*, 2004), analgesic/anti-inflammatory activity (Del *et al.*, 2004), antioxidant activity (Scheller, 1989; Majiene *et al.*, 1996, 2004; Yong, 1998), immunoenhancement activity (Volpi, 2004), anti-tumor activity (Ikukatsu *et al.*, 2002; Murata *et al.*, 2004), and anti-allergic activity (Aso *et al.*, 2004).

MATERIALS AND METHODS

Original samples of Agaricus produced in Brazil, were supplied by Shizenkyosei Corp

A powdered mixture *Propolis* of these three substances prepared using an agate mortar. For the extraction, *Propolis* (100 g) was powdered, and 300 ml of 70% ethanol was added. After drying at room temperature, filtration was carried out using folded filter paper. The filtrates were dried using a evaporator. Dried *Propolis* was obtained by freezing and thawing (yield: 53.5 g, recovery: 53.5%).

Male ICR Mice, purchased from Japan Clea Inc., were used at 4 weeks of age. After preliminary feeding for one week, these mice were used for experiments. Animals were housed in the animal facility at 23 ± 10°C humidity, 55 ± 5% with a 12 h light-12h dark cycle. The animals had food (Japan Clea Inc., CE-2) and water ad libitum.

Antioxidant activity of the simple constituent *Propolis* measurement of DPPH radical scavenging activity

To examine radical scavenging activity, we measured the reactivity of DPPH, a stable free radical as a model of unsaturated fatty acid radicals.

DPPH (1,1-diphenyl-2-picrylhydrazyl), MES buffer (2-morpholinoethanesulfonic acid, monohydrate) and ethanol were purchased from Wako Pure Chemical Industries, Inc. Trolox, used as positive standard material, was purchased from Aldrich Chemical Co. Inc., and 400 μM DPPH, 0.2 M MES buffer, 80% ethanol, 20% ethanol and 0.2 mM Trolox were prepared. All reagents were commercially available special grades.

A mixture consisting of 15 ml of 400 μM DPPH, 15 ml of 0.2 M MES buffer (pH 6.0) and 15 ml of 20% ethanol was prepared. To 0.9 ml of the mixture, 300 μl of 80% ethanol was added. As test samples, 0.20% *Propolis* was prepared, and then 0, 30, 60, 90, 120 and 150 μl of samples were serially added at intervals of 30 seconds. The solution was stirred using a vortex mixer and the absorbance at 520 nm was serially measured at intervals of 30 seconds and 20 min after adding the samples. Trolox, which was positive standard material and a water-soluble derivative of tocopherol, was adjusted to concentrations as low as 0.2 mM, and added in the

order of 0, 30, 60, 90, 120 and 150 μl (Orsolic *et al.*, 2003; Gach *et al.*, 2005). Then, 4.7 ml of 75% ethanol, 0.1 ml of 30% Rhodan ammonium and 0.1 ml of 0.2 mM ferrous chloride in 3.5% HCl were added to the reaction mixture after 1, 3, 5 and 7 days, and absorbance at 500 nm was measured after 3 min (Chang *et al.*, 2005; Chen *et al.*, 2005; Kuo *et al.*, 2005).

Lymphocyte versus polymorphonulcear leukocyte ratio enhancement activity (L/P activity)

The Flescher *et al.* modification of the method of Glinsky *et al.* was used. Immunologically immature neonatal litters of Swiss-Webster mice (Japan SLC, Inc.), within 6 - 12 hr after birth were divided into two groups. In the control group, 0.02 ml of saline was injected intraperitoneally, and in the sample groups, *Propolis* (200 μg/mouse) was injected in 0.02 ml intraperitoneally. Blood was collected from the tail vein before injection, and 6, 10 and 14 days after injection. Thin-layer blood smears were prepared and stained by Wright's staining method. The number of lymphocytes and polymorphonuclear leukocytes were counted, so that the total cell number was 100, using a microscope with a mechanical stage, and the ratio of the number of lymphocytes to the number of polymorphonuclear leukocytes (L/P ratio) was calculated Efficacy was evaluated according to the method of Mizutani *et al.* (1978) A P value < 0.05 by t-test was considered statistically significant.

Anti-tumor activity

Anti-tumor activity in Ehrlich solid carcinoma

Ten male ICR mice (Japan SLC, Inc.) were used at 4 weeks of age per group. Twenty-four hours after transplantation of Ehrlich solid carcinoma (1×10^6 cells, 0.05 ml), distilled water (10 ml/kg), *Propolis* (80 mg/kg body weight B.W.) was administered orally for 34 days. The size of the tumor (long length × short length × thickness, in mm^3) was measured using a micrometer five times every other week for 35 days. Tumors were removed 35 days after transplantation, and the weight of the tumor (g) was measured. Inhibitory ratio in tumor growth (IR) was calculated according to the following formula.

Anti-tumor activity in Sarcoma-180 solid carcinoma

Ten male ICR mice (Japan SLC, Inc.) at 4 weeks of age were used per group. Sarcoma-180 solid carcinoma (1×10^6 cells, 0.05 ml) was transplanted into the right inguinal region subcutaneously. Twenty-four hours after transplantation, distilled water (10 ml/kg B.W.) was administered orally to the control group, and *Propolis* (80 mg/kg B.W.) was administered orally to the other groups for 34 consecutive days. The size of tumor was measured by measuring the length of the largest diameter using a micrometer from the surface of the skin five times every other week from 7 days to 35 days after transplantation. At 35 days after transplantation, tumors were removed, and the weight (g) of tumors were measured. The inhibitory ratio in tumor growth compared with the control group treated with distilled water was calculated according to the formula described above.

Mechanism affecting immunoresponse of tumor-bearing mice

Measurement of interferon gamma (IFN-γ)

An ELISA (enzyme-linked immunosorbent assay) method was used for the measurement of IFN-γ using a mouse IFN-α ELISA kit (Amersham Biosciences, Inc.).

Ten male ICR mice at 4 weeks of age were used per group. Sarcoma-180 solid carcinoma (1×10^6 cells) was transplanted into the right inguinal region subcutaneously. Distilled water (10 ml/kg B.W.) was administered orally to the control group, and *Propolis* (80 mg/kg B.W.) was administered orally to the sample groups Four weeks after administration, 5 ml of PBS was injected into the mice intraperitoneally. After rubbing the abdominal region, peritoneal fluid was collected. The resulting peritoneal fluid was frozen at -20°C for 3 hr. After defrosting the frozen fluid, the supernatant obtained

by centrifugation (3000 rpm, 10 min) was transferred to a dialysis membrane. The solution was concentrated using polyethylene glycol (#4000) and used as a sample. Fifty micro liters of the sample and the standard were distributed to each well and incubated at room temperature (20 - 25°C) for 120 min after covering with a plate cover. Then, 50 μl of biotinylated antibody reagent was added to each well, and incubation was carried out at room temperature (20 - 25°C) for 60 min. After washing three times with wash buffer, 100 μl of streptavidin-HRP incubation medium was added and wells were covered with a plate cover. After incubation at room temperature for 30 min, washing was carried out three times with wash buffer. One hundred micro liters of TMB solution was added and incubation was carried out for more than 30 min. Incubation times were determined according to the extent of development of blue color.

After incubation, 100 μl of stop solution was added. Absorbance at 450 nm was measured using a Labsystems Multiskan MS-UV (Dainippon Pharmaceutical Co., Ltd) within 30 min and the amount of IFN-γ was estimated based on absorbance using a standard calibration curve.

Measurement of tumor necrosis factor-alpha (TNF-α)

Measurement of TNF-α was carried out by an ELISA method using a mouse TNF-α ELISA kit (Pierce Biotechnology, Inc.).

Ten male ICR mice at 4 weeks age were used per group. Sarcoma-180 solid carcinoma was transplanted into right the inguinal region subcutaneously. Distilled water (10 ml/kg B.W.) was administered orally to the control group, and *Propolis* (80 mg/kg B.W.) was administered orally to the experimentsl groups. Four weeks after administration, 5 ml of PBS was injected intraperitoneally. After rubbing the abdominal region, peritoneal fluid was collected. The resulting peritoneal fluid was frozen at -20°C for 3 hr. After defrosting the frozen fluid, the supernatant obtained by centrifugation (3000 rpm, 10 min) was transferred to a dialysis membrane. The solution was concentrated using polyethylene glycol (#4000) and used as a sample. Fifty micro liters of the sample and the standard were distributed to each well and incubated at room temperature (20 - 25°C) for 120 min after covering with a plate cover. Then, 50 μl of biotinylated antibody reagent was added to each well, and samples were incubated at room temperature (20 - 25°C) for 2 hr. After washing five times with wash buffer, 100 μl of HRP solution was added and wells were covered with a plate cover. After incubation at room temperature (20 - 25°C) for 30 min, washing was carried out five times with wash buffer. One hundred micro liters of TMB solution was added and incubation was carried out for more than 30 min. Incubation time was determined according to the extent of development of blue color. After incubation, 100 μl of stop buffer was added. Absorbance at 450 nm was measured using a Labsystems Multiskan MS-UV (Dainippon Pharmaceutical Co., Ltd) within 30 min after addition of stop buffer and the amount of TNF-α was estimated based on absorbance using a standard calibration curve.

Measurement of IgM

IgM was measured by an ELISA method using a mouse IgM ELISA quantitation kit (Bethyl Laboratories, Inc., catalog No.: E90-101) and an ELISA starter accessory package (Bethyl Laboratories, Inc., catalog No.: E101).

Ten male ICR mice at 4 weeks of age were used per group. Sarcoma-180 solid carcinoma (1×10^6 cells) was transplanted into the right inguinal region subcutaneously. Distilled water (10 mg/kg B.W.) was administered orally to the control group, and *Propolis* (80 mg/kg B.W.) was administered orally into the sample groups. Four weeks after administration, blood was collected from the eyeground. Resulting serum was used as sample. Affinity-purified antibody was diluted 100-fold with coating buffer and capture antibody solution

was prepared. One hundred micro liters of solution was transferred to each well, and incubation was carried out for 60 min. Capture antibody solution was then aspirated, and each well was washed twice with wash solution. Next, 200 μl of postcoat solution was added to each well, and incubation was carried out for 30 min. Postcoat solution was then aspirated, and washing was carried out twice with wash solution. One hundred micro liters of sample diluted 500-fold and mouse reference serum prepared by dilution with standard diluent were transferred into each well, and incubation was carried out for 60 min. After washing the well four times with wash solution, 100 μl of antibody/HRP conjugate in conjugate diluent, which was prepared by diluting goat anti-mouse IgM-Fc-HRP 120000-fold with sample/conjugate diluent, was transferred into each well. After incubation for 60 min, wells were washed 4 times with wash solution. Finally, 100 μl of substrate solution (TMB) was added to each well. After incubating for 10 min, the reaction was stopped by adding 100 μl of 2 M H_2SO_4 to each well, and absorbance at 405 nm was measured using a Labsystems Multiskan WS-UV (Dainippon Pharmacetutical Co., Inc.).

Measurement of IgG

IgG was measured by an ELISA method using a mouse IgG ELISA quantitation kit (Bethyl Laboratories, Inc., catalog No.: E90-131) and an ELISA starter accessory package (Bethyl Laboratories, Inc., catalog No.: E101).

Ten male ICR mice at 4 weeks of age were used per group. Sarcoma-180 solid carcinoma (1×10^6 cells) was transplanted into the right inguinal region subcutaneously. Distilled water (10 ml/kg B.W.) was administered orally to the control group, and *Propolis* (80 mg/kg B.W.) was administered orally to experimental groups. Four weeks after administration, blood was collected from the eyeground and serum was used as samples.

Capture antibody solution was prepared by diluting affinity-purified antibody 100-fold with coating buffer. One hundred micro liters of the resulting solution was transferred to each well. After incubation for 60 min, capture antibody solution was aspirated, and each well was washed twice with wash solution. Next, 200 μl of postcoat solution was added to each well. After incubation for 30 min, postcoat solution was aspirated, and the well was washed twice with wash solution. In addition, 100 μl of the sample diluted 1000-fold and the standard prepared by diluting mouse reference serum with standard diluent were transferred to each well. After incubation for 60 min, the well was washed four times with wash solution. Then, 100 μl of antibody/HRP conjugate in conjugate diluent, which was prepared by diluting goat anti-mouse IgG-Fc-HRP conjugate 90000-fold with sample/conjugate diluent, was transferred to each well. After incubation for 60 min, the well was washed four times with wash solution. Finally, 100 μl of substrate solution (TMB) was added to each well, and incubation was carried out for 7 min. The reaction was stopped by adding 100 μl of 2 M H_2SO_4, and absorbance at 450 nm was measured using a Labsystem Multiskan MS-UV (Dainippon Pharmacetutical Co., Inc.).

RESULTS

Antioxidant activity

Results of DPPH radical scavenging activity

With respect to radical scavenging activity, water of which had no radical scavenging activity, showed no marked difference. *Propolis* showed greater radical scavenging activity than 0.2 mM Trolox, which was used as a positive standard.

Lymphocyte versus polymorphonuclear leukocyte enhancement activity (L/P activity)

Ten neonatal Swiss-Webster mice were divided into two groups. Saline was injected intraperitoneally into one group, and *Propolis* was injected intraperitoneally into the other group at a dose of 200 μg/mouse, as shown in Fig. 3. The arrow (↑)

Table 1. Polymorphonuclear leukocytes ratio increase promotion action vs lymphocytes

Sample	Dose (μg/mouse)	Group	Number of Mouse	Days after the injection		
				6 days	10 days	14 days
	200	Control	5	0.50 ± 0.03	0.89 ± 0.07	1.22 ± 0.15
	200	*Propolis*	5	0.78 ± 0.09**	1.95 ± 0.28**	2.55 ± 0.39**

**Statistically significant ($P < 0.01$) from the control group. In the control group, 0.02 ml of saline was injected intraperitoneally, and in the sample groups, *Propolis* (200 μg/mouse) was injected in 0.02 ml intraperitoneally. Blood was collected from the tail vein before injection, and 6, 10 and 14 days after injection.

Table 2. Test materials for Ehrlich solid cancer of antitumor effects by oral administration of *Propolis*

Sample	Dose (mg/kg B.W. ×days PO)	Administration Days	Number of mice	Tumor size (g)	Inhibition rate %
Control	10 × 34	1P-34	10	5.31 ± 0.98	-
Propolis	80 × 34	1P-34	9	3.76 ± 0.88*	29.1

*Statistically significant ($P < 0.05$) from the control group. 24 hours after transplantation of Ehrlich solid carcinoma (1×10^6 cells), distilled water, *Propolis* was administered orally for 34 days. The size of the tumor (long length x short length x thickness, in mm^3) was measured using a micrometer five times every other week for 35 days.

Table 3. Test materials for Sarcoma180 carcinoma cells of antitumor effects by oral administration of *Propolis*

Sample	Dose (mg/kg B.W. × days PO)	Administration Days	Number of mice	Tumor size (g)	Inhibition rate %
Control	10 × 34	1-34	10	7.05 ± 1.57	-
Propolis	80 × 34	1-34	9	2.64 ± 1.37*	29.1

*Statistically significant ($P < 0.05$) from the control group. Sarcoma-180 solid carcinoma (1×10^6 cells) was transplanted into the right inguinal region subcutaneously. Twenty-four hours after transplantation, distilled water (10 ml/kg) was administered orally to the control group, and *Propolis* (80 mg/kg) was administered orally to the other groups for 34 consecutive days.

shows the day of injection. Significant differences from the control group ($P < 0.01$) were shown 6, 10 and 14 days after injection.

As shown in Table 1, L/P ratios after the administration of *Propolis* (200 μg/mouse) were 0.78 ± 0.09 (day 6), 1.95 ± 0.28 (day 10) and 2.55 ± 0.39 (day 14), whereas L/P ratios in the control group were 0.50 ± 0.03 (day 6), 0.89 ± 0.07 (day 10) and 1.22 ± 0.15 (day 14). The L/P ratio after the administration of *Propolis* was significantly elevated ($P < 0.01$), as compared to the control group.

Results of anti-tumor activity

Anti-tumor activity in Ehrlich solid carcinoma

With respect to anti-tumor activity in Ehrlich solid carcinoma, when *Propolis* (80 mg/kg B.W.) was administered for 34 consecutive days, suppressive ratios were 29.1% ($P < 0.05$), as shown in Table 2.

Anti-tumor activity in Sarcoma-180 solid carcinoma

In Sarcoma-100 solid carcinoma, when *Propolis* (80 mg/kg B.W./day) were orally administered for 34 consecutive days, suppressive ratios were 62.6% ($P < 0.05$), as shown in Table 3.

Mechanism affecting *Propolis*-induced immunoresponse to tumor-bearing mice

Results of IFN-γ activity in the mice bearing Sarcoma-180.

A mouse IFN-γ ELISA kit (Amersham Bioscience) was used for the measurement of IFN-γ and the results are shown in Table 4. The horizontal axis

Table 4. Sarcoma-180 carcinoma mouse on the IFN-γ of producing ability

Sample (mg/kg.B.D)	IFN-γ (pg/mouse)
	Mean ± S.D.
Control	10.19 ± 5.25
Propolis	18.51 ± 5.16*

*Statistically significant ($P < 0.05$) from the control group. Absorbance at 450 nm was measured using a Labsystems Multiskan MS-UV (Dainippon Pharmaceutical Co., Ltd) within 30 min and the amount of IFN-γ was estimated based on absorbance using a standard calibration curve.

Table 5. Sarcoma-180 carcinoma mouse on the TNF-α of producing ability

Sample (mg/kg B.W.)	TNF-α (pg/mouse)
	Mean ± S.D.
Control	62 ± 14.77
Propolis	61.61 ± 11.16

Absorbance at 450 nm was measured using a Labsystems Multiskan MS-UV (Dainippon Pharmaceutical Co., Ltd) within 30 min after addition of stop buffer and the amount of TNF-α was estimated based on absorbance using a standard calibration curve.

and vertical axis indicate each group and IFN-γ, respectively.

Results of TNF-α activity in the mice bearing Sarcoma 180

A mouse TNF-α ELISA kit (Pierce Biotechnology) was used for the measurement of TNF-α and the results are shown in Table 5. The horizontal axis and vertical axis indicate each group and TNF-α, respectively.

Results of IgM activity in the mice bearing Sarcoma-180

A mouse IgM quantitation kit (Bethyl Laboratories, Inc., catalog No.: E90-101) and an ELISA starter accessory package (Bethyl Laboratories, Inc., catalog No.: E101) were used for the measurement of IgM and the results are shown in Table 6. The horizontal axis and vertical axis indicate each group and IgM, respectively.

Table 6. Sarcoma-180 carcinoma mouse on the IgM of producing ability

Sample (mg/kg B.W.)	IgM (ng/ml)
	Mean ± S.D.
Control	113.86 ± 3.95
Propolis	132.91 ± 7.35**

**Statistically significant ($P < 0.01$) from the control group. The reaction was stopped by adding 100 μl of 2 M H_2SO_4 to each well, and absorbance at 405 nm was measured using a Labsystems Multiskan WS-UV (Dainippon Pharmacetutical Co., Inc.).

Table 7. Sarcoma-180 carcinoma mouse on the IgG of producing ability

Sample (mg/kg B.W.)	IgG (ng/ml)
	Mean ± S.D.
Control	200.81 ± 9.82
Propolis	291.44 ± 14.09**

**Statistically significant ($P < 0.01$) from the control group. The reaction was stopped by adding 100 μl of 2 M H_2SO_4, and absorbance at 450 nm was measured using a Labsystem Multiskan MS-UV (Dainippon Pharmacetutical Co., Inc.).

Results of IgG activity in the mice bearing Sarcoma-180

A mouse IgG ELISA quantitation kit (Bethyl Laboratories, Inc., catalog No.: E90-101) and an ELISA starter accessory package (Bethyl Laboratories, Inc., catalog No.: E101) were used for the measurement of IgG and the results are shown in Table 7. The horizontal axis and vertical axis indicate each group and IgG, respectively.

DISCUSSION

When the antioxidant activities of *Propolis* were examined by the DPPH and Rhoudan iron methods, all samples showed radical scavenging activity. *Propolis* showed antioxidant activity. Chen *et al.* (1990) reported that flavonoids showed high antioxidant activity. Since *Propolis* contains high levels of flavonoids, it is thought that flavonoids may be responsible for the antioxidant activity in this study (Scheller *et al.*, 1989; Majiene *et al.*, 1996, 2004).

With respect to lymphocyte versus polymorphoculear leukocyte enhancement activity, it is known that lymphocytes act to specifically recognize pathogenic bacteria and induce an adaptive immunoresponse. B cells recognize particular antigens via receptors on the cell surface and produce antibodies to eliminate the antigens. T cells undergo antigen presentation, and are classified into helper T cells (Th1 and Th2 are involved in cellular immunity and humoral immunity, respectively), cytotoxic T cells, inflammatory T cells and suppressor T cells × (Anderson *et al.*, 2005). In the examination of immunoenhancement activity, we measured lymphocyte versus polymorphonuclear leukocyte ratios (L/P activity). The number of lymphocytes was significantly increased in groups treated with *Proplolis*. The reason that the immature immunoresponse of neonatal mice was activated to the same level observed in mature mice might be because various constituents in the samples used in this experiment activated the neonatal immune system.

IFN-γ is a multi-functional factor which shows antiviral effects, suppression of cell growth, anti-tumor effects, activation of macrophages, enhancement of NK cell activation, regulation of immunoresponse, and regulation of the induction of differentiation. Specifically, slightly high levels of IFN-γ were measured in mice bearing the S-180 carcinoma, after administration of *Propolis*. This strongly suggests that cellular immunity is especially activated by treatment with *Propolis*, because production of IFN-γ is limited to the T cells and NK cells stimulated by mitogen and sensitized antigen (Kalinski *et al.*, 2005).

TNF-α shows a different extent and mechanism of action depending on the target cells. TNF-α causes impairment and suppression of growth in tumor cells, enhancement of growth, expression of IL-2 receptor and enhancement of IFN-γ and CSF production in T cells, and induction of antibody production in B cells. In addition, TNF-α increases cytotoxic activity in NK cells and LAK cells. In the macrophage, TNF-α increased cytotoxicity and enhances production of PGE2, IL-1, 6, 8, CSF and active oxygen (Hirose *et al.*, 2004). When TNF-α was measured in mice bearing the S-180 carcinoma, mice treated with *Propolis* showed slightly higher TNF-α levels as compared to the control group. This suggests that activated macrophages produce TNF-α in mice treated with *Propolis*, since activated macrophages and lymphocytes are the source of most TNF-α.

When macrophages act as antigen-presenting cells (APC), they present antigens to helper T cells which give an auxiliary signal of stimulation to B-cells via the CD40 ligand. At the same time, helper T cells activate B-cells by producing cytokines. At this time, the direction of class switching of B cells is determined and differentiation to plasma cell occurs. IgM is an antibody with a pentamer structure and its production precedes IgG production in response to an antigen. Distinctive features of IgM include 10 antigen binding sites, easy production in response to granular antigens such as red blood cells, bacteria and viruses, and strong aggregation activity and activation of the complement system. Mice bearing a carcinoma and treated with *Propolis*, showed higher levels of IgM than the control group. This could be the result of enhancement of B cell activation in all treated groups, because immature B cells express antigen receptor, IgM on cell surface by binding of CD40 ligand located on the membrane of activated helper CD4 T cell to CD40 molecule located on the membrane of B cell (Duhen *et al.*, 2004).

IgG is abundant in the blood, and causes a marked enhancement of phagocytosis by phagocytes and macrophages, although the extent is different in each subclass. In mice bearing S-180 carcinoma, groups treated with *Propolis* showed higher IgG production than control groups. IL-4 is mainly associated with a class switch of IgM to IgG. IFN-γ and IL-4 are related to differentiation of Th1 and Th2, respectively, and these cytokines suppress each other (Liou *et al.*, 2004). Therefore, after treatment with *Propolis*, which resulted in a high

production of IFN-γ, differentiation to Th2 is suppressed, and a significant class switch to IgG doesn't occur. Thus, Th1-related cellular immunity might be activated.

When anti-tumor action was examined using two kinds of sarcoma (Ehrlich solid carcinoma and Sarcoma-180 carcinoma), tumor-suppressive ratios after treatment with *Propolis* was 29.1%. When Sarcoma-180 solid carcinoma was used, tumor-suppressive ratios were 62%. Thus, *Propolis* showed strong anti-tumor activity against two kinds of solid carcinoma. Taken altogether, this strongly suggests that *Propolis* enhances original functions of macrophages and NK cells, and as a result, secondarily enhances the immune reaction and suppresses tumor growth. On the other hand, significant increases in TNF-α suggest an enhancement of T cell growth and induction of cytotoxic activity towards tumor cells and a suppression of tumor growth (Zuo *et al.*, 2001). Thus, high anti-tumor activity would be expressed more effectively due to direct activity towards tumor cells and activation of a host-mediated immunoreaction. *Propolis* shows anti-tumor activity, there is the possibility that *Propolis* restores the balance between NF-κB and I-κB, and induces the apoptosis and tumor suppression secondarily (Lin *et al.*, 2004).

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