Effects of a Plum (Prunus mume Siebold and Zucc.) Ethanol Extract on the Immune System In Vivo and In Vitro

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The effect of a plum ethanol extract (PEE) on immunity was analyzed. An oral administration of PEE increased the interleukin (IL)-12p40 concentration in the serum and T-cell ratio in the spleen. In vitro studies revealed that PEE stimulated IL-12p70 production in peritoneal macrophages and natural killer activity. These findings suggest that PEE enhanced the immune function by stimulating innate immune cells.

Key words: plum ethanol extract; innate immunity; immune enhancement; interleukin-12; ripeness

The plum (Prunus mume Siebold and Zucc.) is a deciduous tree of the genus Rosaceae that is native to China and other eastern Asian countries and has long been popular as a raw material included in herbal preparations in traditional Korean medical preparations as a remedy for coughs and dyspepsia.¹ It has recently been reported that plums possessed such beneficial biological activities as blood fluidity improvement,² anti-fatigue action,³ anti-oxidative and free radical scavenging activities,⁴ inhibiting the motility of Helicobacter pylori⁵ and protection from Helicobacter pylori⁶ and the human influenza A virus.⁷ Previous reports on anti-microbial activity prompted us to investigate the immune function. We demonstrate for the first time in this report that an ethanol extract prepared from plum affected innate immunity both in vivo and in vitro.

We prepared the plum ethanol extract (PEE) by immersing for one month 1 kg of green or ripe plums, produced in Nankou (Wakayama, Japan), in 1.8 kg of 40% ethanol; the whole ethanol extract was then freeze-dried to yield 44.2 g of PEE powder. We examined the in vivo effect on the immune function of PEE derived from green plums after feeding 6-week-old female C57BL/6J mice (Charles River Japan, Kanagawa, Japan) for 4 d with 4 g/d of an AIN-93 pellet containing PEE. A 10 mg/head amount of PEE was fed on the first day for acclimatization, and 50 mg/head was fed for the remaining days. The control group was fed 4 g/d of an AIN-93 pellet. Each diet was placed daily in the cages and confirmed to have been fully consumed. The mice were sacrificed on day 4 for collection of the blood and spleen. There were no significant differences in body weight or the ingested amount between the control and PEE-fed groups. This experiment was performed in accordance with the guidelines for the care and use of laboratory animals of Kirin Holdings Co., Ltd. (Tokyo).

To determine the effect of PEE administration on the systemic innate immune system, the concentration of plasma IL-12p40, a pro-inflammatory cytokine mainly produced by antigen-presenting cells (APCs),⁸ was evaluated by an enzyme-linked immuno-sorbent assay (ELISA, OptEIA Mouse IL-12(p40) ELISA kit, BD Pharmingen). Figure 1A shows that the plasma IL-12p40 concentration was significantly higher in the PEE-fed group than in the control group (p < 0.05). It has been reported that IL-12 was produced by activated macrophages or dendritic cells;⁹ our data therefore suggest that the oral administration of PEE affected the innate immune response by activating APCs and promoting IL-12p40 production in the serum.

The ratio of T lymphocytes in the spleen was evaluated to determine the effect of PEE administration on the acquired immunity. Total splenocytes were stained with anti-CD3ε, anti-CD4 and anti-CD8α antibodies and analyzed by flow cytometry. Interestingly, the ratio of CD3+ T lymphocytes in the PEE-fed group was significantly higher than that in the control group (p < 0.05; Fig. 1B). The ratios of both CD4+ and CD8+ T cells in the PEE-fed group were also significantly higher than those in the control group (Fig. 1B). It has been reported that up-regulation of IL-12 led to effective stimulation of naïve T cells;¹⁰ the increase in T lymphocytes in the spleen might therefore have been due to the activation of innate immune cells.

In vitro studies were performed to determine whether the active compounds in PEE which elicited immune stimulation were polar or non-polar substances. Since ripe plum and green plum may differ in their compositions, both ripe and green plum-derived PEE samples were investigated. Freeze-dried PEE was dissolved in 10% methanol and applied to a Mega BE-C18 cartridge (10 g, 60 mL; Varian). The cartridge was washed with 45 mL of 10% methanol (polar fraction), and the adsorbed material was then eluted with 20 mL of 100% methanol (non-polar fraction). Each fraction was dried by rotary evaporation and stored at −20°C before being used. The collection rate for the non-polar fraction in green plums was 74.8% and that for the polar fraction was 15.4%, while the collection rate for the non-polar fraction in ripe plums was 82.7% and that for the polar-

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Abbreviations: PEE, plum ethanol extract; IL-12, interleukin-12; APC, antigen-presenting cell; ELISA, enzyme-linked immuno-sorbent assay
fraction was 12.8%. It is well known that APCs and natural killer cells (NK cells) play a major role in innate immunity; we therefore focused on the effects of PEE on macrophages and NK cells.

We examined the effects of fractionated PEE on macrophages in vitro by culturing for 24 h thioglycollate-induced peritoneal macrophages with or without 100 μg/mL of PEE and with 5 ng/mL of lipopolysaccharide (LPS from Salmonella typhosa). The medium for cell culture was RPMI 1640 (Sigma-Aldrich, St. Louis, MO, USA) containing 100 U/mL of penicillin, 100 mg/mL of streptomycin (Sigma-Aldrich), 1% non-essential amino acid (Gibco, Grand Island, MI, USA), 1% non-essential amino acid (Gibco), 1 mmol/L of sodium pyruvate (Gibco) and 10% fetal calf serum (FCS; Hyclone, Logan, UT, USA). The IL-12p70 concentration in the medium was measured after 24 h. The result shown is representative of three independent experiments.

Fig. 1. Effects of PEE on the Immune System in Vivo.
Mice (n = 3) were fed with or without PEE for 4 d and then sacrificed for collection of the plasma and spleen. A, Concentration of IL-12p40 in the plasma; B, Ratio of the T lymphocyte subpopulation (%). Each value represents the mean ± SD. Data for the control group (unfilled bars) and PEE-fed group (hatched bars) are shown. Statistical comparisons were performed by using Student’s t test. Significant differences compared to the control, *: p < 0.01, #: p < 0.05.

Fig. 2. Effect of PEE on Peritoneal Macrophages.
Thioglycolate-induced macrophages were cultured for 24 h with or without 100 μg/mL of fractionated PEE and with 5 ng/mL of lipopolysaccharide (LPS from Salmonella typhosa). The medium for cell culture was RPMI 1640 (Sigma-Aldrich, St. Louis, MO, USA) containing 100 U/mL of penicillin, 100 mg/mL of streptomycin (Sigma-Aldrich), 5 × 10^{-3} mol/L of 2-mercaptoethanol (Gibco, Grand Island, MI, USA), 1% non-essential amino acid (Gibco), 1 mmol/L of sodium pyruvate (Gibco) and 10% fetal calf serum (FCS; Hyclone, Logan, UT, USA). The IL-12p70 concentration in the medium was measured after 24 h. The result shown is representative of three independent experiments.

The IL-12p70 concentration was measured by culture supernatants with or without 100 μg/mL of PEE, and with or without 5 ng/mL of LPS from Salmonella typhosa (Sigma-Aldrich). The concentration of IL-12p70 in each supernatant was measured by ELISA (an OptEIA Mouse IL-12(p70) ELISA kit, BD Pharmingen). The concentration of IL-12p70 was increased when fractionated PEE was added (Fig. 2). This effect did not differ between PEE from green plums and ripe plums. In contrast, no IL-12p70 was produced when LPS was not added with or without the PEE fraction (data not shown). Moreover, the concentration of IL-12p70 was not increased when the cells were cultured either with citric acid, which was the main component of the polar fraction, or with amygdaline, a component of the non-polar fraction (data not shown). It has been reported that such groups of polyphenols and flavonoids as mumefural, lyoniresinol, and syringaresinol are present in plums.2,12) These compounds would have been eluted in the non-polar fraction, so that plum-derived polyphenols and flavonoids may be involved in elevating the IL-12p70 production from peritoneal macrophages. Our data indicate that more than two compounds in PEE, apart from citric acid and amygdaline, had IL-12p70 induction activity in macrophages in the presence of a low volume of LPS. The activity observed in the polar fraction may also be related to polysaccharides, and another possibility is that the active compounds responsible for immune stimulation present in the PEE extract may not have been completely fractionated into the non-polar and polar fractions.

We examined the effects of fractionated PEE on NK cells in vitro by culturing splenocytes for 24 h with or without 100 μg/mL of PEE. Subsequently, 1 × 10^6 splenocytes were cultured for 4 h with 1 × 10^5 Yac-1 cells (Riken, Tsukuba, Japan) which are known to be the targets of NK cells. We next examined the cytotoxic activity of NK cells by measuring the concentration of LDH in the culture medium with a Cytotoxicity Detection kitPLUS(LDH) (Roche). The cytotoxic activity was increased when fractionated PEE was added (Fig. 3), although the cytotoxic activity of the polar and non-polar fractions of PEE made from green plums did not differ. However, the cytotoxic activity of the polar fraction of PEE made from ripe plums was more effective than both the non-polar fraction of PEE made from ripe plums and the polar fraction of PEE made from green plums (Fig. 3). Our data indicate that more than two compounds in PEE activated the NK cell activity, and that these compounds might have been affected by the ripeness of the plums.

Previous work has indicated that plums have a number of beneficial biological activities. We have demonstrated in this study that PEE may have enhanced the innate immunity in vivo and in vitro, as assessed by the level of IL-12 concentration and other factors. We have also shown that an oral administration of PEE could enhance the adaptive immunity in vivo, as assessed by the ratio of T lymphocytes in the spleen.
It has been reported that T lymphocytes play an important role in host defense against pathogens,13) so that the increase of T lymphocytes in the spleen should lead to enhanced host defense against pathogens. Jung et al. have shown that fermented maesil (i.e., plum) had an immunity enhancing effect on mice,14) and we suggest that some compounds, which were not produced by fermentation, could affect the immunity enhancing activity in vivo (Fig. 1). We also suggest that the level of ripeness is important for the activity of natural killer cells (Fig. 3). Taken together, we suggest that PEE affected the immune cells by contributing to host defense against pathogens and could provide health benefits. PEE contains a number of compounds, both in the polar and non-polar fractions; the major components of PEE responsible for its immunity enhancing effect are still unclear and studies are currently in progress to identify these.

References