

National Formosa University
Department of Biotechnology
Service Commission Project Report

Project Number: 105AF002

Project Name: The immunomodulatory effect of
Prunus mume fruit

Requester (Institute): YOU DE LTD

Implementation period: 105/05/01~105/07/31

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Abstract

Prunus mume (*P. mume*) is used as a health food or medicinal material in traditional herb medicine for a long time in Taiwanese, Japanese, and Korean. The fruit of it exhibits antioxidant, anti-inflammation, anti-Viruses, anti-bacteria, anti-cancer, and free radical scavenging activity. Recent project report (number: NFMI 105001) found that concentrated powder of *Prunus mume* fruit from YOU DE LTD has anti-cancer activity. Higher the concentration of concentrated *P. mume* fruit powder solution, more cancer cell can be killed. Until now, the immunomodulatory effect of it is still unknown.

Dendritic cells (DCs) are now recognized as the most potent professional antigen presenting cells (APCs) involved in initiating primary immune responses. They play a central role in the initiation and regulation of immune responses. DCs modulation strategies for improving appropriate immune function are being developed for preventing or treatment cancers and infectious diseases.

In this study, bone marrow derived DCs were co-cultured with concentrated powder of *P. mume* fruit (0.1, 1, 10 or 20 µg/mL). CD11c, CD80, CD86, and MHCII surface markers were analyzed by flow cytometry and cytokine production were evaluated by ELISA kit. Our results demonstrated that concentrated powder of *Prunus mume* fruit from YOU DE LTD unable promote dendritic cell maturation and IL-10/ IL-12 cytokine secretion. Although more study is needed, the findings are suggested that this product can be used to modulate immunity.

KEYWORDS: Dendritic cell, Immunomodulatory, IL-12, IL-10, *Prunus mume*

Introduction

Prunus mume

Prunus mume (*P. mume*) is the deciduous trees of the rose family. The fruit of this tree is used in food and traditional medicine. *P. mume* originated in China, and it was later introduced to South Korea and Japan. According to compendium of materia medica “Ben cao gang mu”, fruit of *P. mume* possesses various pharmacological activities. One study reported that ingredient named 5-hydroxymethylfurfural (HMF) in fruit of *P. mume* has anti-allergic effect. Other reports also showed that anti-oxidant activity (Hoshino et al. 2013) and anti-cancer activity by the other two ingredients MK615 (Sunaga 2012; Tada et al. 2012) and C19H22O6 (Jeong et al. 2006). It seems that *P. mume* not only has high ornamental value but also has effect on healthcare and pharmaceutical use.

Dendritic cells; DCs

Dendritic cells circulated in the blood and then residence as immature form in the skin, mucous membranes, and organs such as the lungs and spleen. Direct recognition of invading pathogens activates immature DCs and induces their differentiation. DCs are most potent antigen-presenting cells for directing innate and adaptive immunity. After DCs become mature, they subsequently present antigen peptide to priming CD4 + T cells (Th cells). DC maturation can show significant up regulation of surface markers (CD40, CD80, CD86, MHC-II) and instruct distinct programs of T helper cell differentiation depending on the type of polarizing cytokines involved (eg, IL-12 for Th1, IL-4 for Th2, IL-10 for Tr1, IL-6 for Th17, and TGF β for Treg) (Ito et al. 2007). Naïve Th cell possess immune mechanisms and finally generation of memory T cells (Geiss et al. 2016). Surface markers of DCs such as CD80, CD86 highly expression and IL-12 secretion can promote Th1 polarization, low surface marker expression of CD80, CD86 and IL-10 secretion lead to Tr1 development. If DCs don't highly express the surface protein CD80, CD86, MHC II and do not secrete cytokine IL-12, TNF- α , it will lead T cell to loss functions and to apoptosis (Walsh et al. 2004). It means that DCs when they unmaturred DCs can induce Treg to secrete IL-10 and TGF- β (Jonuleit, et al. 2000).

Interleukin-12 (IL-12)

Interleukin-12 (IL-12) has an essential role in the interaction between the innate and adaptive arms of immunity (Trinchieri, 1995). DCs can phagocytose pathogens then secrete IL-12. IL-12 acts on T helper cells (Th cells), NK cells, and cytotoxic T cells (Tc cells). (Colombo et al. 2002). The anti-tumor efficacy of IL-12 has been demonstrated in animal models of cancer of diverse types (Simpson et al. 2009; Denies et al. 2014).

Interleukin-10 (IL-10)

Immature DCs that produced significant amounts of IL-10 display low surface expression of MHC class I and II molecules and T cell costimulatory molecules of the B7 family (Mahanke et al. 2016). The major role of IL-10 is to limit the extent of innate and adaptive immune reaction and to maintain an immune homeostatic state (Maynard et al. 2008; Roncarolo et al. 2006). IL-10 can protect infection-associated immunopathology, autoimmunity, allergy, and so on. IL-10 regulates growth or differentiation of B cells, NK cells, cytotoxic and helper T cells, mast cells, granulocytes, dendritic cells, keratinocytes, and endothelial cells (Moore et al. 2001). Thus IL-10 would be instrumental in the future development of the immune therapeutic potentials (MacKenzie et al. 2014; O'Garra et al. 2008).

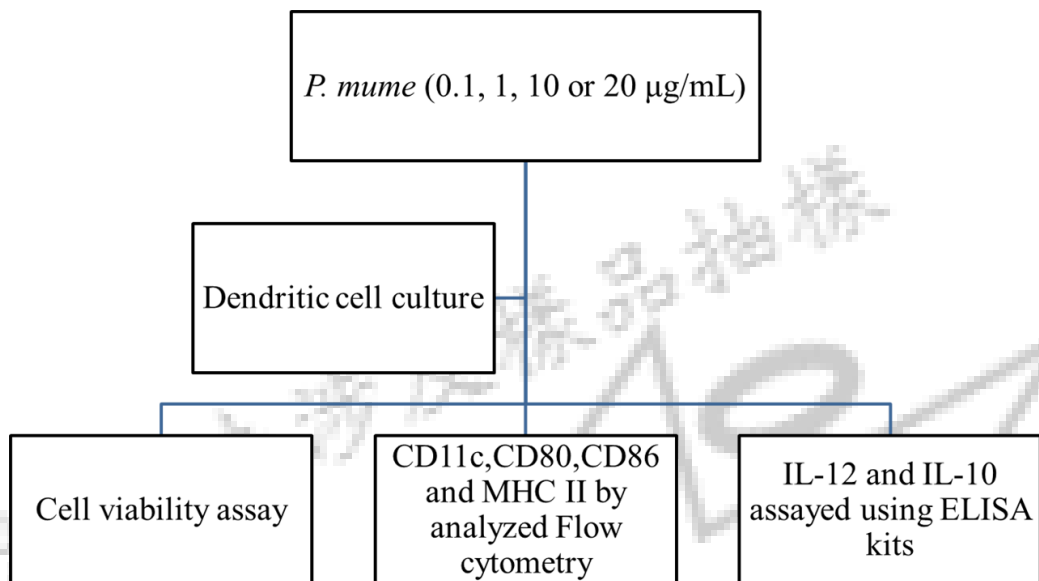
Immunomodulatory effect

Immunomodulatory effect also referred to as immunotherapy activity especially indicated for the immune regulation activity of candidate drugs. If some drugs can be used to re-establish a suitable state of the immune system, it will help doctors to regulate the overreaction such as allergy and autoimmune, or can also be used to enhance a better immune response to anti-cancer. By enhancing Th1 immune function or promoting Treg immune tolerance are two different strategies to modulate immune response. The regulation of dendritic cell activation will affect the polarization direction of the follow-up T helper cells.

Specific aim of this study

Cancer therapy strategies such as chemical, radiation and surgical control accompanied with a great harm for human body. Immunotherapy for anti-cancer becomes to new strategy in recent years. Promote dendritic cell maturation by natural compound and strengthening the immune reaction is one promising way to anti-cancer. In this study, we tested the concentrated power of *P. mume* fruit on mouse bone marrow-derived DCs. The immunomodulatory effect of it is investigated.

Experimental design



Experimental materials

Mice

Female BALB/c mice (3-6 weeks of age) were purchased from National Laboratory Animal Center (Taiwan). The animal room was maintained on a 12-h light/dark cycle with a constant temperature ($23 \pm 2^\circ\text{C}$) and humidity. Animal care and handling conformed to the *NIH Guide for the Care and Use of Laboratory Animals*. All experiments were performed under protocols approved by the Biotechnology Department of National Formosa University affidavit of approval of animal use protocol (Animal approval number A105-02).

Experimental equipment :

Centrifuge	(5922 Microprocessor, Kubota, Japan)
Thermostatic circulator	(Digisystem, Germany)
Microscope	(Nikon, Japan)
CO ₂ Incubators	(Heal force, Hong Kong)
UV/VIS Spectrophotometer	(Metertech)
Flow cytometry	(BD)

Consumable material :

Cryogenic Vial	(Corning®, Mexico)
hemocytometer	(Marienfeld, Germany)
24well	(Nunc Tm , Denmark)
10 ml pipette	(Falcon, USA)
Centrifuge tube 15 、 50ml	(Falcon, USA)

Experimental Drugs :

Antibiotic	(Biological Industries, Israel)
Roswell Park Memorial Institute-1640 (RPMI-1640)	(Sigma, USA)
Fetal bovine serum (FBS)	(Biological Industries, Israel)
Glucose	(Riedel-de Haën, Seelze)
HEPES	(PanreacAppliChem, USA)
L-glutamin	(Biological Industries, Israel)
Non-essential amino acid (NEAA)	(Biological Industries, Israel)
Soudium Bicarbonate	(Riedel-de Haën, Seelze)

Sodium Pyruvate	(Biological Industries,Israel)
Trypan blue	(Biological Industries,Israel)
Concentrated <i>P. mume</i> fruit powder	(provided by YOU DE LTD)

Experimental kit

Mouse IL-10	ELISA Kit	(R&D)
Mouse IL-12	ELISA Kit	(R&D)

Fluorescent Antibody

Anti-Mouse CD86 (B7-2) PE	(ebioscience)
Anti-Mouse CD11c FITC	(ebioscience)
Anti-Mouse CD80 (B7-1) PE	(ebioscience)
Anti-Mouse MHC Class II (I-A/I-E) PE	(ebioscience)
Rat IgG2a K Iso type CONTROL PE	(ebioscience)
Mouse IgG1 K Iso type Control FITC	(ebioscience)

Methods

Bone marrow derived dendritic cell culture

Mice were sacrificed by dislocated. Feet were removed and then removed all muscle from the femurs and tibiae bones were separated and, cut both ends of femurs and tibiae bones carefully using sterile, sharp scissors to expose the interior marrow. Flushed the contents of marrow with 3 ml of RPMI-1640 complete medium using a 10 ml syringe with a 25G needle.. Seeded cells on 24-well culture plates in RPMI 1640 complete medium supplemented with 5% heat-inactivated FBS and 10 ng/mL mouse GM-CSF for 6 days. For DC maturation, BMDC were cultured in 24-well plates for 4 days and treated with *P. mume* at the indicated concentrations. After 48 hours, stained with mAb against CD11c, CD80, CD86 and MHC II and analyzed by flow cytometry.

Cell Viability

After treated with *P. mume*, take 2 ul of the DCs cell suspension and mixed with an equal volume of 18 ul trypan blue reagent. Hemocytometer was used to observe under an inverted microscope. The number of live cells in hemocytometer was used for calculating the percentage cytotoxicity as % Cytotoxicity = Live Cell treated *P. mume*/Live Cell No. × 100.

ELISA for evaluation of IL-12, IL-10

Supernatants were collected after treated with *P. mume*. Then IL-10 and IL-12 were measured using ELISA kits from R&D System. Briefly, 96-well ELISA plates were coated with the capture antibody and incubated at room temperature overnight. The wells were blocked with blocking buffer (1% bovine serum albumin) [BSA], in PBS with 0.05% NaN₃) to prevent nonspecific binding. The cell supernatants were added, and samples were incubated for 2 h. After incubation, the plates were then washed with washing buffer (0.05% Tween 20 in PBS, pH 7.2–7.4). The detection antibody was added for an additional 2h, and plates were washed again with washing buffer. Avidin-horseradish peroxidase (HRP) was then added, and plates were incubated for 20 min at room temperature before washing. Finally, substrate solution (1:1 mixture of H₂O₂ and tetramethylbenzidine) was added, the plates were incubated for 20 min at room temperature, stop solution (2N H₂SO₄) was added, and the OD₄₅₀ nm value was measured using an ELISA plate reader.

Statistical analysis

Statistical evaluation was performed by using Student's t tests with SigmaPlotv.12.0 software (Systat Software, San Jose, CA, USA). Differences with p values of less than 0.05 were considered significant, and differences with p values of less than 0.01 were considered highly significant.

Results:

P.mume (0.1 ~ 20 ug / mL) co-cultivated with DCs are some toxic but almost above 80% viability (Figure 1). CD11c, CD86 surface marker expression of DCs significantly suppressed by *P.mume*. These data indicated that *P.mume* can inhibit DCs to be mature (Figure 2). IL-10 and IL-12 do not secrete by DCs. (Data not shown)

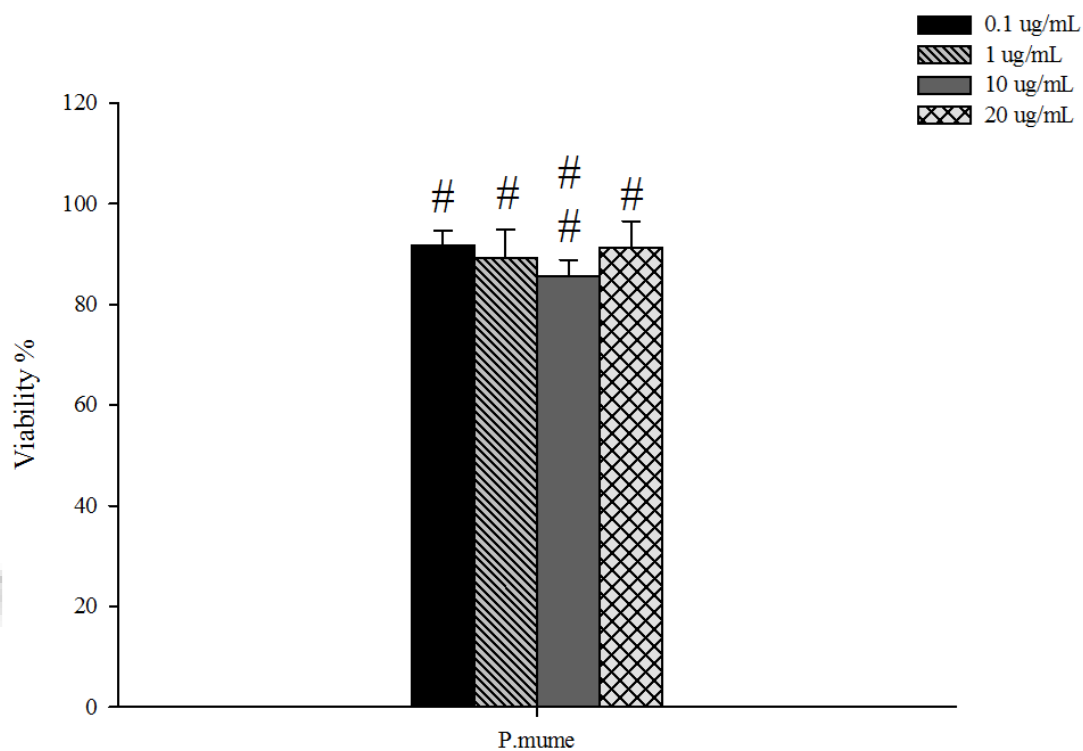


Fig1. The cell cytotoxicity effect of concentrated *P. mume* fruit powder on dendritic cells

Cell viability was performed on dendritic cells after 48 h treatment with increasing concentrations of concentrated *P. mume* fruit powder solution. Data shown are representative of three independent experiments. Error bars indicated mean \pm SD of triplicate samples. The significances * $p < 0.05$, ** $p < 0.01$ when compared to cell viability of no-treated DCs. (Student's t-test)

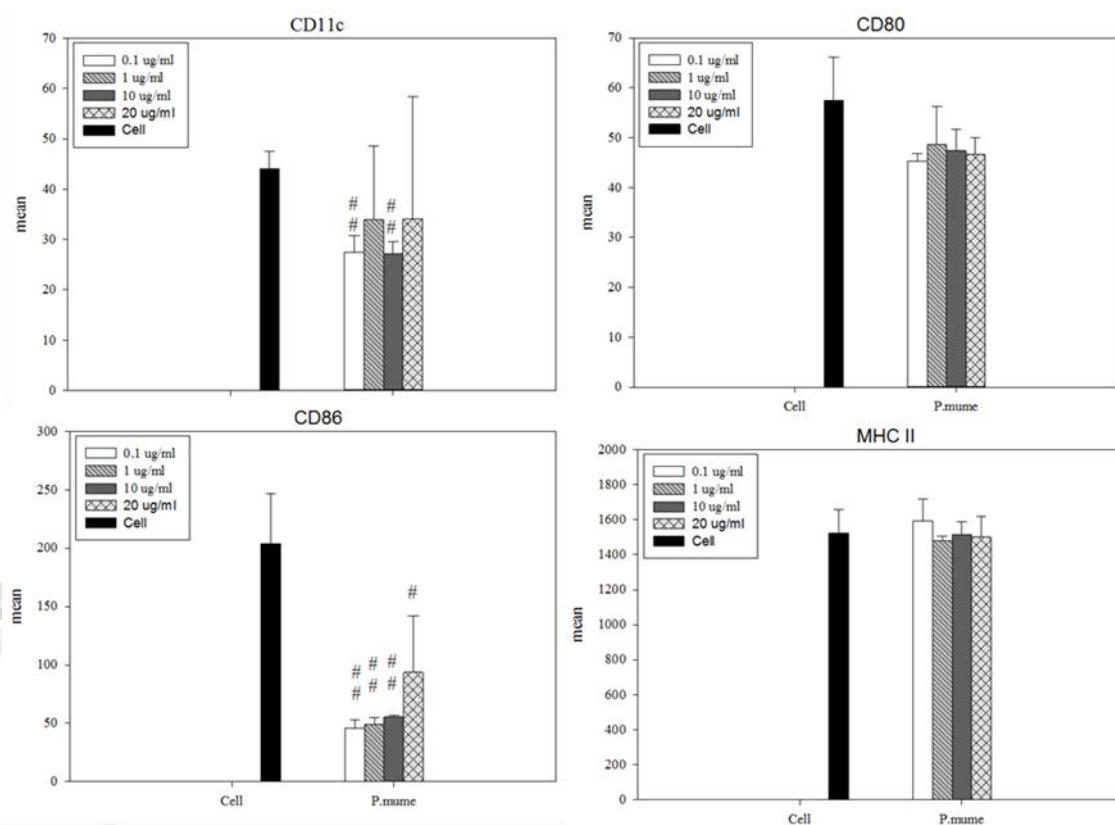


Fig2. The *P. mume* fruit powder effects on BMDC activation

Cell surface markers were performed on dendritic cells after 48 h treatment with increasing concentrations of concentrated *P. mume* fruit powder solution. Data shown are representative of three independent experiments. Error bars indicated mean \pm SD of triplicate samples. The significances * $p < 0.05$, ** $p < 0.01$ when compared to cell viability of no-treated DCs. (Student's t-test)

Discussion

In the study of Tsuji et al., ethanol extracts of *P. mume* *in vitro* and *in vivo* can promote macrophages to secrete IL-12 (Tsuji et al. 2011). The triterpenoids of *P. mume* extract can inhibit inflammatory response by LPS stimulating macrophages through High mobility group box-1 protein (HMGB1) suppression and increased the antioxidant effects of Nrf2 / HO-1(Kawahara et al. 2009). The cell types used in these two studies are different from cells we used in this study. In our study, we found that *P. mume* cannot promote DCs maturation and IL-10/ IL-12 cytokine secretion. Further investigation is needed to evaluate the immunomodulatory effects of it .

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