

## Effects of Jujube Fruit Extract on Peripheral Blood Mononuclear Cell Proliferation, Cytokine Productions and Intracellular Hydrogen Peroxide Level

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### Abstract

**Background:** *Ziziphus* genus (jujube) is a fruit, which is commonly found and used in Eastern traditional medicine for various conditions. Immunomodulation and anti-oxidant activities of jujube fruit extract are reported. This study aimed to observe immunomodulation effects of green (unripe) and ripe jujube fruit extract on peripheral blood mononuclear cell (PBMC) proliferation by carboxyfluorescein diacetate (CFSE) fluorescence, cytokine profiling by enzyme-linked immunosorbent assay (ELISA), and intracellular H<sub>2</sub>O<sub>2</sub> level by dichlorodihydrofluorescein diacetate (DCFH-DA) fluorescence. **Results:** Treatments of PBMC with both green and ripe pulp extracts resulted in non-significant increases, about 1.01 - 1.15 times, in PBMC proliferation compared to the untreated group ( $p > 0.05$ ). The pro-inflammatory cytokine (IL-1 $\beta$ ) and regulatory Th2 cytokine (IL-10) levels were significantly higher in PBMC treated with ripe jujube fruit extract than in the cells stimulated with green jujube extract ( $p < 0.05$ ). Th1 cytokine (IFN- $\gamma$ ) level was not significantly different between the ripe and green jujube extract. A higher percentage of intracellular H<sub>2</sub>O<sub>2</sub> reduction was observed in PBMC treated with the green pulp extract compared to the ripe pulp extract ( $p < 0.05$ ). **Conclusions:** This study provides practical information about an effect of jujube staging on some cytokine inductions. The reduction of intracellular oxidative stress seems to involve these cytokine induction phenomena.

**Keywords:** Jujube, immune, cytokines, intracellular hydrogen peroxide

### Introduction

The genus *Ziziphus* is one of the most widespread members of the Rhamnaceae family, called jujube. According to traditional medicine, the extract of bark and wood of *Ziziphus atropensis* Pierre has been commonly used as a tonic, carminative, appetizer, and muscle analgesic [1]. *Z. jujuba* Mill. is most commonly found in Thailand. *Z. mistol* helps to prevent the development of complications related to oxidative stress generated in the blood of patients with Hemolytic Uremic Syndrome-producing *Escherichia coli* [2].

Jujube fruit cultivar *Z. jujuba* contains 2 types of cytokinin-like compounds when tested using the soybean callus test. Zeatin is a compound found in jujube by paper and column chromatography and coupled with various physicochemical tests techniques [3]. Moreover, the extracts of *Z. jujuba* cv. *jinsixiaozao* Hort consist of at least 2 pectic polysaccharides, Ju-B-2 and Ju-B-3. Ju-B-2 induced mice

spleen cells proliferation in a dose dependent manner, while, Ju-B-3 did not [4]. Immunomodulatory activities of *Z. jujuba* leaf extracts were investigated by neutrophil locomotion and Nitro Blue Tetrazolium test in human neutrophils. They found that various concentrations of water and alcoholic parts of *Z. jujuba* extracts stimulated cell-mediated immune response by increasing neutrophils function and phagocytic activity [5]. Furthermore, high doses (100 - 400 mg/kg body weight) of water extract from the seeds of *Z. jujuba* also stimulated both cell-mediated immune response and humoral immune response in mice after measuring anti-SRBC titer, delayed type hypersensitivity response, nitroblue tetrazolium reduction activity, nitric oxide synthase activity and bactericidal activity, and IFN and IL-4 levels. The effects were equivalent to a standard drug (Levamisole, 2.5 mg/kg body weight). These findings suggest a possible mechanism that is related to the function of macrophage and Th-1 cells [6].

There are many different types of cells in the immune system. Most lymphocytes play a role in the secretion of cytokines to encourage a variety of other cells in the immune response. Cell function in the immune system associated with at least 2 types of T helper lymphocytes are T helper-1 (Th1) and T helper-2 (Th2). Th1 cells secrete cytokines such as IL-2, IL-3, IL-12, IFN- $\gamma$  and TNF- $\alpha$  and acts on cell-mediated responses. At the same time, Th2 cells secrete IL-3, IL-4, IL-5, IL-6, IL-10 and IL-13 and act on the humoral immunity response [7]. These types of cytokines are associated with increased cell proliferation and cell function in the immune system. This study measured the impact of the water extract of jujube fruit on peripheral blood mononuclear cell (PBMC) proliferation and cytokine production. The factors that affect the impact of jujube extracts on PBMC function were also investigated including oxidative status in PBMC.

Reactive oxygen species (ROS) are important in regulating the response of cells. Low concentrations of ROS stimulate proliferation, differentiation, migration and cell death. ROS are generated by electron transport processes in cells under normal conditions. This includes free radicals such as superoxide ( $O_2^{\bullet-}$ ), hydroxyl radical ( $OH^{\bullet}$ ), peroxy ( $ROO^{\bullet}$ ) and non-radicals; hydrogen peroxide ( $H_2O_2$ ) and singlet oxygen ( $^1O_2$ ) [8].  $O_2^{\bullet-}$  is a free oxygen radical and is the primary ROS of electron transport in cells. Secondary ROS including  $H_2O_2$ , which is the active substance in the reaction and stable, can be measured easily [9]. The over generated free radicals can cause oxidative stress within the cell and result in biomolecular damage (such as DNA, protein, lipids and small cellular molecules). Oxidative damage relates to cells function and survival. The immune cells are, therefore, affected by oxidative stress [10]. This study aims to evaluate the immunomodulatory effect of jujube extract including immune cells proliferation, cytokine profiling and intracellular  $H_2O_2$  level for understanding the effect of green and ripe jujube on immune cells function.

## Materials and methods

### Jujube extraction

Both green fruit and ripe fruit of a common cultivar (Samros) in Thailand were collected from local markets during 2011 - 2012. The maturity of the samples were chosen according to consumer-acceptance standards and harvesting details as previously described [11]. Briefly, fresh pulps of jujubes were separated from the seeds and were macerated in water. The mixtures were dry-frozen (Freeze dryer, G MMA 1-1 LS, CHRIST, Osterode, Germany). The lyophilized water extracts were dissolved with 50 % aqueous ethanol (ratio 1:10, w/v). Then the mixture was centrifuged at  $1400\times g$  for 20 min and the pellet was re-extracted [12]. The 2 supernatant water extracts of jujubes were combined and used for PBMC treatment for the immunomodulation study including immune cells proliferation measurement by CFSE fluorescence, cytokine profiling assay by ELISA, and intracellular  $H_2O_2$  level measurement by DCFH-DA fluorescence.

### Isolation of the PBMC

PBMC was isolated as previously described [13]. This study was approved by the Khon Kaen University Ethics Committee (HE552271). Briefly, venous blood was drawn from a healthy volunteer and diluted twice with PBS. PBMC were isolated by centrifugation on Ficoll-Hypaque gradient (density 1.077 g/mL) and centrifuged at  $400\times g$  for 35 min. The interphase cells, consisting of PBMC were then washed

3 times with a culture medium. The cells were re-suspended in a culture medium, consisting of RPMI-1640, supplemented with 10 % fetal calf serum, L-glutamine, antibiotics and mitogens 5 ng/mL lipopolysaccharide and 1 µg/mL concanavalin A at a density of  $1 \times 10^6$  cells/mL.

#### **PBMC proliferation assay**

Succinimidyl ester of carboxyfluorescein diacetate (CFSE) (Molecular Probe, USA) is an intracellular protein covalent coupling dye that can be used to track cell division. PBMC were stained with 10 µM of CFSE dye at 37 °C for 30 min. Then, they were washed 3 times with 10 % FBS/RPMI and  $1 \times 10^6$  of stained cells were placed in a 24 well plate. Jujube extracts were then incubated to PBMC for 24 h and the cells were measured for fluorescence intensity with a flow cytometer. After cell division, halving of the fluorescence intensity of dye can be detected in each divided cell [14,15].

#### **Cytokines detection by ELISA**

After 24 h of jujube extracts incubation with PBMC, the supernatant was collected and kept at -20 °C for cytokines detection. The levels of IL-1β, IL-10 and IFN-γ were measured by Enzyme linked immunosorbent assay (ELISA) which is the specific immunoassay between antibody and the antigens of interest [16]. The assay was performed following the manufacturer's instructions of commercial Human IL-1 beta ELISA Ready-SET-Go, Human IL-10 ELISA Ready-SET-Go and Human IFN gamma ELISA Ready-SET-Go (eBioscience, USA). The absorbance was measured by a spectrophotometer at 450 nm and a reference wavelength at 570 nm.

#### **Intracellular hydrogen peroxide detection by fluorescence probe assay**

After PBMC were treated with jujube extracts for 24 h, the cells were washed with PBS and pelleted by centrifugation. The resuspended cells in PBS were incubated with 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) at room temperature for 30 min. DCF fluorescence was instantly analyzed by flow cytometry [17]. The green fluorescence produced is thus proportional to the H<sub>2</sub>O<sub>2</sub> produced.

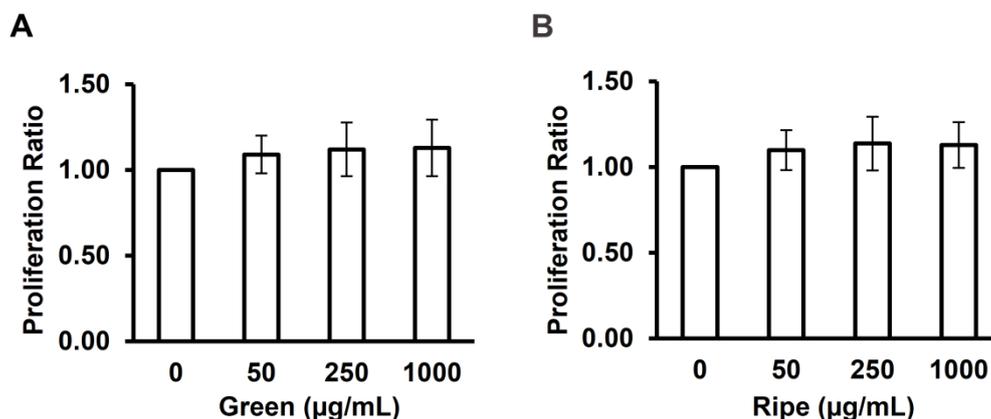
#### **Data analysis**

SPSS was performed to analyze statistical differences between cells treated with jujube extracts and untreated group (one-way ANOVA). Statistical significance was considered within the 95 % confidence interval when  $p < 0.05$ .

### **Results and discussion**

#### **Effect of jujube water extract of ripe and green jujubes on immune cells proliferation**

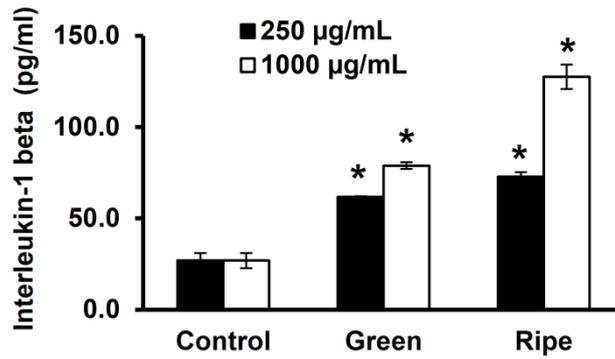
The proliferation of PBMC cells were determined by CFSE fluorescence dye. CFSE will be divided when cell are divided to 2 cells. Percentage of cells with decrease in fluorescence intensity were observed and can be measured by flow cytometer [14,15]. The results showed that both green pulp and ripe pulp extracts slightly increased PBMC cells proliferation from 1.01 - 1.15 times as compared to the untreated group (**Figure 1**), with no significant difference found ( $p > 0.05$ ). The water extract of green and ripe jujube fruit affected immune cells or peripheral blood mononuclear cell (PBMC), which consists of T lymphocyte, B lymphocyte, NK lymphocyte and monocyte. These types of cells can secrete various cytokines, cytokines that are associated with the increasing number and functions of immune cells. Moreover, jujube extracts could stimulate immune cells response by increasing the secretion of cytokines from PBMC and tended to increase PBMC proliferation. The increment of PBMC proliferation was not significant after treatment with either green or ripe jujube extracts. This is in contrast to the findings of Yu *et al.* [18] that the extracts of crude polysaccharides fraction, deproteinized polysaccharides fraction, oligosaccharide fraction and ammonia water fraction from Chinese jujube (*Z. jujuba*) can stimulate proliferation of the mouse spleen white blood cells under inflammation conditions stimulated by *Euphorbia kansui* and prostratin [18]. The difference may involve the status of the immune cells because our study was done under the normal conditions of human immune cell, without inflammatory induction.



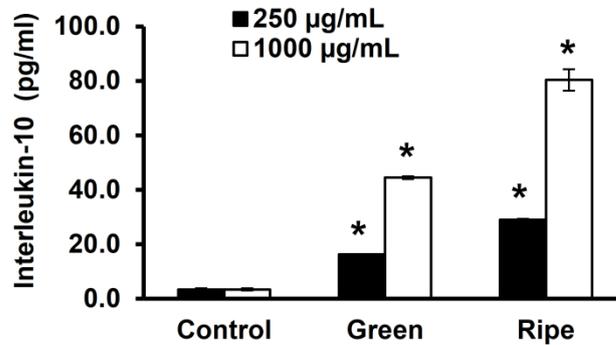
**Figure 1** PBMC proliferation measured after 50, 250 and 1,000 µg/ml of jujube water extracts treatment compared to the untreated group. Proliferation ratio determined by comparing fluorescence intensity to untreated controls. The jujube extracts treatment showed a slight induction effect on PBMC cells proliferation from 1.01 - 1.15 ratio by green (a) and ripe Samros (b). Both extracts were not significantly different ( $p > 0.05$ ).

#### Effect of jujube water extract of ripe and green jujubes on cytokines secretion from immune cells

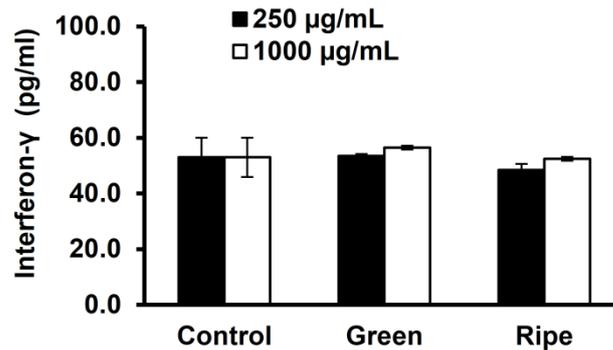
To investigate the effects of the jujube extract on immune cells function, the levels of cytokines associated with Th1 lymphocytes (IFN- $\gamma$ ) or the Th2 lymphocytes (IL-10) and pro-inflammatory (IL-1 $\beta$ ) were measured. We found that both green and ripe jujube showed an increase in IL-1 $\beta$  and IL-10 from treated PBMC by dose dependent manner (250 and 1,000 µg/ml). A higher IL-1 $\beta$  secretion level was found for the ripe fruit treatment (127.5 pg/ml) compared to the green fruit treatment (78.9 pg/ml) (**Figure 2**). A higher IL-10 secretion level was also found for the ripe fruit treatment (80.4 pg/ml) compared to the green fruit treatment (44.5 pg/ml) (**Figure 3**). However, no significant effect on the level of IFN- $\gamma$  was observed (**Figure 4**). Similar observation regarding an increase in IL-1 $\beta$  and IL-10 was previously reported in a garlic derivative (alliin) anti-oxidant compound. This substance can also stimulate PBMC proliferation and increase IL-1 $\beta$  production [19]. Furthermore, our finding is consistent with the effects of Chinese medicine formula (CKMB) on immune cells. CKMB contain 5 types of herbs including *Z. jujube*. CKMB could activate the immune cells by increasing the secretion of IL-1 $\beta$ , IFN- $\gamma$ , IL-10, TNF- $\alpha$  and IL-6 (20, 21). IL-1 $\beta$  is a pro-inflammatory cytokine and IL-10 is involved in the development process (differentiation) of T-lymphocyte to Th2, which is involved in the humeral immune response [7,20]. Th1 mediated cytokine, IFN- $\gamma$ , did not influence the response of Th1. The data are different from the seed jujube extract (*Z. jujuba* from India) that increased the secretion of IFN- $\gamma$  by lymphocytes [21]. However, the PBMC used in this study were from healthy humans which were different from that reported by Mishra and Bhatia [21], which were reported in mouse splenocytes [21].



**Figure 2** Interleukin-1 $\beta$  (pg/ml) secretion level of PBMC treated with green and ripe jujube water extracts at 250 and 1,000  $\mu\text{g/ml}$  for 24 h was measured by ELISA. Both green fruit and ripe fruit water extracts exerted the increasing of IL-1 $\beta$  from treated PBMC by dose dependent manner (250 and 1,000  $\mu\text{g/ml}$ ) (\* $p < 0.05$ ).



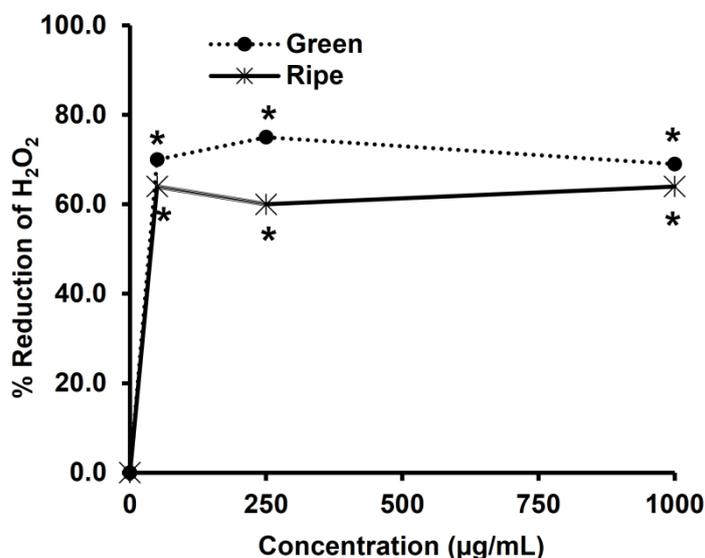
**Figure 3** Interleukin-10 (pg/ml) secretion level of PBMC treated with green and ripe jujube water extracts at 250 and 1,000  $\mu\text{g/ml}$  for 24 h was measured by ELISA. Both green fruit and ripe fruit water extracts exerted the increasing of IL-10 from treated PBMC by dose dependent manner (250 and 1,000  $\mu\text{g/ml}$ ) (\* $p < 0.05$ ).



**Figure 4** Interferon- $\gamma$  (pg/ml) secretion level of PBMC treated with green and ripe jujube water extracts at 250 and 1,000  $\mu\text{g/ml}$  for 24 h was measured by ELISA. Both green fruit and ripe fruit water extracts showed no significantly increasing of Interferon- $\gamma$  from treated PBMC as compared to control group ( $p > 0.05$ ).

### Effect of jujube water extracts on intracellular hydrogen peroxide level in immune cells

The 50, 250 and 1,000  $\mu\text{g/ml}$  of both green and ripe jujube treated PBMC for 24 h showed a significant reduction in the intracellular  $\text{H}_2\text{O}_2$  level ( $p < 0.05$ ). A higher reduction of the intracellular  $\text{H}_2\text{O}_2$  level in PBMC was found in the green fruit treatment than the ripe fruit treatment ( $p < 0.05$ ) (Figure 5). However, the reduction of  $\text{H}_2\text{O}_2$  levels in PBMC cells showed no correlation with dose dependence. This study was to investigate the effect of the jujube water extraction on PBMC cells using  $\text{H}_2\text{O}_2$  levels as an intracellular oxidative stress indicator. The previous data reported that the methanol extract of jujube showed high anti-oxidative effects of free radicals. Many phenolic compounds such as catechin, caffeic acid, epicatechin, ferulic acid, rutin, *p*-hydroxybenzoic acid and chlorogenic acid are found in jujube [22]. The extracts contained flavonoid and catechin, a phenolic compound which can convert  $\text{H}_2\text{O}_2$  into water by electron transfer [23,24]. The phenolic compounds were detected in both green and ripe fruit which are consistent with the previous report. Both green or ripe fruit cultivars can reduce intracellular  $\text{H}_2\text{O}_2$ , but this effect is not related to the concentration of jujube extract.



**Figure 5** Intracellular  $\text{H}_2\text{O}_2$  level in PBMC cells treated with 50, 250 and 1,000  $\mu\text{g/ml}$  green and ripe jujube extract for 24 h was detected by DCFH-DA. The significant higher reduction of intracellular  $\text{H}_2\text{O}_2$  level in PBMC was found at green fruit treatment than ripe fruit treatment as compared to control group ( $p > 0.05$ ).

### Conclusions

Both green and ripe fruit Jujube extracts showed immunomodulatory effects via controlling the secretion of pro-inflammatory cytokines (IL-1 $\beta$ ) and Th2 cytokine (IL-10) from immune cells. Although, their tend to activate PBMC cells proliferation is still unclear, more analysis may provide answers. Intracellular oxidative stress was reduced by both green or ripe fruit, higher reduction was found in the green fruit. Oxidative stress may be involved in induction of these cytokines.

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