



Composition analysis and immuno-modulatory effect of okra (*Abelmoschus esculentus* L.) extract

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ARTICLE INFO

Article history:

Received 25 May 2011

Received in revised form 3 January 2012

Accepted 25 March 2012

Available online 3 April 2012

Keywords:

Okra
Abelmoschus esculentus L.
Polysaccharide
Dendritic cell
Cytokine

ABSTRACT

The aim of this study was to analyse the composition of okra (*Abelmoschus esculentus* L.) extract and investigate the effect of *A. esculentus* L. polysaccharides (AE-PS) on the maturation and function of dendritic cells (DCs) derived from rat bone marrow hematopoietic cells (BMHCs) *in vitro*. BMHC-derived immature DCs (BMHC-imDCs) were extracted from rats and treated with AE-PS. The hydrolysed okra extract contained 0.6% β -1, 3-D-glucan. AE-PS induced the presence of polymorphic nuclei and elongated protrusion in the BMHC-imDCs, indicating DC activation. Treatment with 100 μ g/mL of AE-PS increased the MHC class II and CD80/86 expression levels by 41% and 42%, respectively. Treated cells had reduced endocytosis activity. The secretion of IL-12 and IFN- γ increased significantly by 120% and 75%, respectively, when treated with 100 μ g/mL of AE-PS. Moreover, IL-10 production was reduced by 66%. In conclusion, AE-PS exhibits stimulatory effects on rat dendritic cells and promotes the secretion of T_H1 cytokines.

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1. Introduction

Okra (*Abelmoschus esculentus* L.) is a perennial plant native to Africa and has been a part of the diet in various parts of the world (Lamont, 1999). Okra is beneficial to the digestive system due to its high content of polysaccharides and microelements. Numerous studies have explored the effects of carbohydrate polymers extracted from different sources on the immune system. β -glucan shows antitumor activity by activating macrophages and natural killer cells through surface receptor binding (Mizuno et al., 1995). A polysaccharide preparation (200 kDa; β -(1–3)-D-glucan) from *Flammulina velutipes* promotes mouse spleen cell proliferation and suppresses growth of the Sarcoma 180 tumour cell line (Leung, Fung, & Choy, 1996). An increasing body of research (Sakagami et al., 1993; Wang, Liu, Ng, Ooi, & Chang, 1995) has indicated that polysaccharides exhibit stimulatory effects on the immune system.

The molecular weight, degree of polymerisation, degree of branching, conformation, and glycosidic linkages of the polymers all have roles in the function of the polymers (Kim et al., 2000). Polysaccharides with molecular weights greater than 1.1×10^6 Da or smaller than 2×10^4 Da have antitumor effects and stimulate TNF- α production (Borchers, Stern, Hackman, Keen, & Gershwin, 1999; Falch, Espevik, Ryan, & Stokke, 2000). Glucans with β -(1–3) and β -(1–6) linkages are primarily responsible for the immunomodulatory and antitumor effects (Mueller et al., 2000; Willment,

Gordon, & Brown, 2001). These observations provide preliminary evidence for an in-depth analysis of the active components of polysaccharides.

Carbohydrate polymers activate the innate immune system by interacting with dendritic cells (DCs), which are professional antigen-presenting cells that provide an important bridge between innate and adaptive immunity (Banchereau & Steinman, 1998; Keller, 2001). Derived from the hematopoietic stem cells in bone marrow, immature DCs reside in body tissues to capture foreign antigens. Upon appropriate stimulation, DCs lose their phagocytic ability and migrate to secondary lymphoid tissues to present the captured antigens to T lymphocytes, a process known as maturation (Satthaporn & Eremine, 2001). Mature DCs also express MHC class II molecules and costimulatory molecules, such as CD80/86 and CD40, which are necessary for effective antigen presentation (Keller, 2001; Luft et al., 2002). Depending on the type of antigen captured and the maturation process, DCs secrete cytokines, such as IFN- γ , IL-12, and IL-4, which guide the development of naïve T-cells down the path of cell-mediated or humoral immunity (Banchereau et al., 2000). Several studies have explored the effect of polysaccharides on DCs. The acidic polysaccharides from *Phellinus linteus* stimulate murine DC maturation with a concomitant increase in CD80/86 and MHC class II molecule expression levels (Park et al., 2003). Recently, a study has shown that *Lycium barbarum* polysaccharides upregulate the expression levels of CD11c and MHC class II molecules and enhance IL-12 secretion in murine DCs (Zhu, Zhao, Zhao, & Chen, 2007). The objective of this study was to determine whether okra polysaccharides have a similar stimulatory effect on murine DCs and the potential as a functional food.

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2. Materials and methods

2.1. Rats

Four-week-old male CD (SD) IGS rats were purchased from Bio-Lasco (Taipei, Taiwan) with initial weights between 75 and 125 g. To allow adjustment to the environment, the rats were fed a basal diet for more than a week with free access to water and feed. The animal rooms were kept at 25 °C and 60% humidity with a 12 h light/dark cycle (8 AM–8 PM). All animal experiments were approved by the Animal Ethics Committee at the National Pingtung University of Science and Technology in Taiwan.

2.2. Preparation of crude and hydrolysed okra extracts

To prepare crude okra extracts, okra fruits were purchased from the market and were cleaned, diced and immersed in equal amount of 95 °C ddH₂O for 4 h before centrifugation at 10,000 × g for 10 min at 4 °C. Polysaccharides were precipitated with three-fold volume of 95% alcohol and collected by centrifugation at 10,000 × g for 10 min at 4 °C. An equal volume of acetone was added for decolorization before centrifugation at 10,000 × g for 10 min at 4 °C. Remnant acetone was allowed to evaporate in the hood, and the crude extract was then lyophilised, ground and stored at –20 °C until use.

To prepare hydrolysed okra extracts, 5 mg of the crude extract was dissolved in 25 mL of 0.1 M sodium acetate buffer (pH 5.2), and 50 U of pullulanase (Sigma, St. Louis, MO, USA) was then added to the mixture at 40 °C. The reaction was allowed to proceed for 2 h before termination with 20% trichloroacetic acid (TCA). The supernatant was collected by centrifugation at 10,000 × g for 10 min at 4 °C. Alcohol (80% final concentration) was used for precipitation at 4 °C for 24 h. The precipitated polysaccharides were centrifuged at 10,000 × g for 10 min at 4 °C, and they were then washed three times with 95% alcohol before being lyophilised, ground and stored at –20 °C until use for cell treatment.

2.3. Composition analysis of crude and hydrolysed okra extracts

The concentration of β-1,3-D-glucan in the extracts was determined using an aniline blue assay with some modifications (Young & Jacobs, 1998). Extracts were dissolved in 3 mL of NaOH, and the pH value was adjusted to 11.5. A 50 mM Na₂HPO₄–NaOH buffer (pH 11.5) was then added to a final volume of 10 mL. The extracts (2 mL) were incubated with 0.2 mL of 1 mg/mL aniline blue for 2 h before measuring the fluorescent intensity at 395 (excitation) and 495 nm (emission). Laminarin (Sigma, St. Louis, MO, USA), a type of β-1,3-D-glucan isolated from *Laminaria digita* algae, was used as the standard.

Measurement of the total sugar content was carried out by a phenol sulphuric acid method as previously described (Dubois, Gilles, Hamilron, Rebers, & Smith, 1956). The amount of reducing sugar was analysed using a 3,5-dinitro-salicylic acid method as previously described (Miller, 1959). The absorbance of the sample solution was read at 540 nm. Glucose was used as a standard in both the total sugar content and reducing sugar analyses.

The protein concentration was determined by a protein assay kit (Bio-Rad, Hercules, CA, USA) according the manufacturer's instructions. The endotoxin contents of the hydrolysed extracts were measured by the chromogenic *Limulus amedocyte* lysate kit (Associates of Cape Cod, East Falmouth, MA, USA) with a maximum sensitivity level of 0.25 EU/mL.

2.4. Isolation of rat bone marrow hematopoietic cells (BMHCs)

Bone marrow was extracted with RPMI 1640 containing 5% FBS and 1% penicillin/streptomycin (P/S) from femur bones of sacrificed rats (between 5 and 8 weeks old). The cells were collected and resuspended in RPMI 1640 complete media supplemented with 10% FBS, 1% P/S, 20 ng/mL rGM-CSF (PeproTech, Rocky Hill, NJ, USA) and 20 ng/mL rIL-4 (PeproTech, Rocky Hill, NJ, USA). The cells (4×10^7) were seeded into a 75 cm² culture flask and incubated at 37 °C with 5% CO₂, and the media was changed on the third and sixth day of culture.

2.5. Stimulation of BMHC-derived immature dendritic cells (BMHC-imDCs) with AE-PS

On the sixth day of culture, the cells were collected by centrifugation, resuspended in RPMI 1640 complete media, and seeded into 24-well flat bottom culture plates (2×10^6 cells/well). The BMHC-imDCs were differentiated after 24 h of incubation. The cells were then treated with various concentrations of hydrolysed AE-PS (25, 50, 100, 200, 400, and 800 µg/mL) and 1 µg/mL LPS (as a positive control) for 48 h at 37 °C with 5% CO₂. Cell morphology, surface antigen presentation, cytokine secretion, and phagocytosis were analysed.

2.6. Cell morphology of BMHC-imDCs

The morphology of BMHCs was visualised with Liu's stain, which is comprised of two staining solutions as follows: Liu's A stain (0.5 g/L methylene blue and 1.7 g/L eosin yellow in methyl alcohol) to visualise the cytoplasm, and Liu's B stain (1.3 g/L azure and 1.4 g/L methylene blue) to visualise the nucleus. The cells were placed in a cytospin (Thermo Scientific, Portsmouth, NH, USA), centrifuged at 350 × g for 10 min and stained with Liu's reagent. Cell morphology was observed using a microscope.

2.7. FACS analysis of the BMHC-imDC surface markers

Cells (1×10^6) were collected, washed with FACS buffer (1% BSA and 0.1% sodium azide in PBS) and resuspended in 100 µL of FACS buffer. Fluorescent monoclonal antibodies (2 µL) against CD11c, MHC class II and CD 80/86 surface antigens (eBioscience, San Diego, CA, USA) were added to the cells, and the cells were then incubated in the dark for 30 min. The cells were then washed with FACS buffer, resuspended in 0.5 mL of 3.8% paraformaldehyde (in PBS) and analysed by a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA).

2.8. Endocytosis activity of BMHC-imDCs

Briefly, 4 µL of 1 mg/mL FITC-dextran (Sigma) was added to 96 µL of 1×10^6 cells (in PBS buffer) that were washed three times with PBS buffer. The cells were divided into two tubes. One tube was incubated at 4 °C for 1 h, and the other tube was incubated at 37 °C for 1 h. After the reaction, the cells were washed three times with cold PBS buffer (4 °C) and resuspended in 0.5 mL FACS buffer for analysis. The uptake of FITC-dextran was analysed by cytofluorimetric analysis using a FACScan.

2.9. Cytokine enzyme-linked immunosorbent assay (ELISA)

After stimulating the BMHC-imDCs with various concentrations of AE-PS for 48 h, the supernatants were collected by centrifugation at 800 × g for 10 min at 4 °C and were then stored at –80 °C. IL-12 levels were determined using an IL-12 p40 ELISA

kit (Biosource, Camarillo, CA, USA) according to the manufacturer's instructions, and the levels of IFN- γ and IL-10 were determined using a cytokine assay kit (Bender, Vienna, Austria) according to the manufacturers' instructions.

2.10. Statistical analysis

The data were analysed using SAS software (SAS institute, Cary, NY, USA), and the data were expressed as the means $\pm$ standard deviations. One-way analysis of variance (one-way ANOVA) and Duncan's test were used to determine the statistical significance between groups. Differences were considered statistically significant at $P \leq 0.05$.

3. Results

3.1. Extraction and analysis of okra polysaccharides

Both crude and hydrolysed okra extracts were prepared. As shown in Table 1, the yield for the crude extraction was 1.6% (wet weight). To increase the solubility of the crude extract without breaking the β -1,3 glycosidic linkages important for biological activity (Dong, Yao, Yang, & Fang, 2002), a hydrolysed extract was

prepared by adding pullulanase to the crude extract to hydrolyse the α -1,6 linkages. The yield for the hydrolysed sample was 52.6% (dry weight), which was calculated from the weight of the crude extract.

Because the β -1,3-D-glucan content of the AE-PS may have an important role in its biological activity, the percentage weight was measured using an aniline blue assay. Using laminarin as a standard, the results indicated that the hydrolysed okra extract contained 0.6% β -1, 3-D-glucan (Table 1). The total and reducing sugar contents of the hydrolysed extract were 67.6% and 1.6%, respectively. The extracted AE-PS had endotoxin levels lower than 0.25 EU/mL, excluding the possibility that the stimulatory effect of AE-PS was due to the presence of endotoxins.

3.2. AE-PS induces morphological changes in BMHC-imDCs, indicating signs of activation

To study the effect of AE-PS on BMHC-imDCs, the morphological changes in BMHC-imDCs when treated with aqueous extracts of AE-PS were examined. BMHC-imDCs were obtained by treating BMHCs with rGM-CSF and rIL-4 for 7 days. The immature dendritic cells were then treated with AE-PS, and morphological changes were recorded. As shown in Fig. 1A and B, BMHC-imDCs without

Table 1
Composition analysis of crude and hydrolysed okra extracts.

Extract	Constituent (%)				
	Extraction rate	Laminarin equivalent	Total sugar	Reducing sugar	Protein
Crude	1.58 $\pm$ 0.29	0.49 $\pm$ 0.01 ^a	90.05 $\pm$ 1.10 ^a	2.30 $\pm$ 0.05 ^a	Trace
Hydrolysed	52.60 $\pm$ 0.49	0.60 $\pm$ 0.04 ^b	67.56 $\pm$ 1.51 ^b	1.62 $\pm$ 0.04 ^b	Trace

^{a,b} Data in the same row with different superscripts are significantly different at $P < 0.05$.

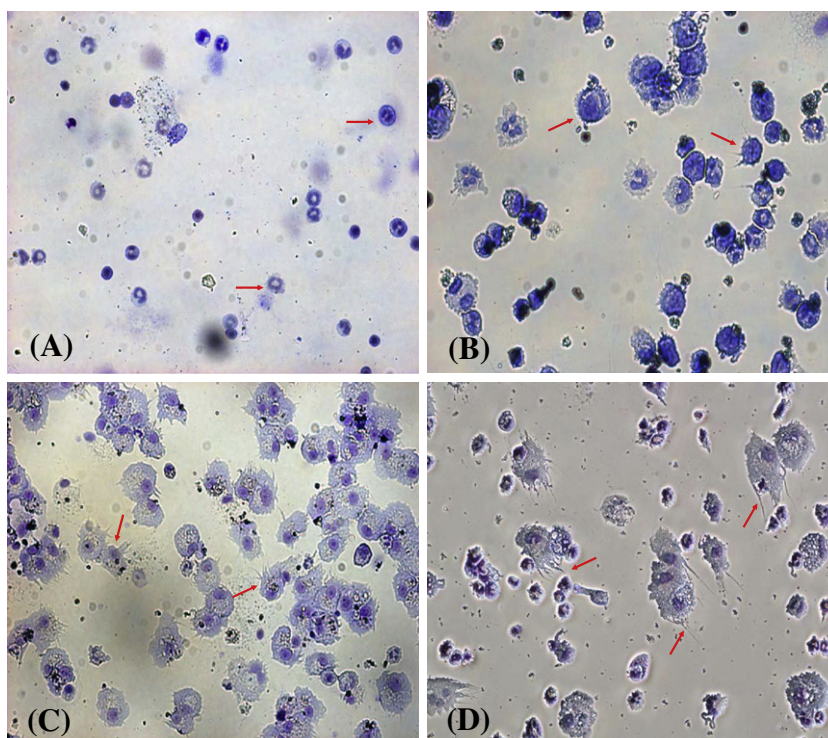


Fig. 1. Morphology of dendritic cells with various treatments. (A) Morphology of bone marrow hematopoietic cells (BMHC) isolated from rats (5 to 8 weeks old) at 200 $\times$ magnification. (B) BMHC immature dendritic cells (BMHC-imDCs) after culturing for 7 days. (C) BMHC-imDCs after stimulation with 1 μ g/mL LPS for 48 h. (D) BMHC-imDCs after stimulation with 100 μ g/mL AE-PS for 48 h.

stimulation appeared round with polymorphic nuclei and small protrusions on the cell surfaces. When treated with 100 $\mu\text{g/mL}$ AE-PS (Fig. 1C) or 1 $\mu\text{g/mL}$ LPS (positive control) for 48 h (Fig. 1D), however, the cells were larger, and the nuclei became even more polymorphic. Moreover, the dendritic protrusions on the cell surfaces became more pronounced and elongated. These features strongly suggested that AE-PS treatment promotes dendritic cell maturation.

3.3. AE-PS stimulates CD11c, MHC class II and CD80/86 expression in dendritic cells

CD11c (LFA-1) was used as a marker for rat DCs (Zhao, Kaneko, Okiji, Takagi, & Suda, 2006) (Fig. 2). FACS analysis revealed that approximately 92.4% of the BMHCs were differentiated into BMHC-imDCs after cytokine treatment. Upregulation of MHC class II and CD80/86 molecules are indicators of dendritic cell activation. MHC class II molecules are important for antigen presentation, and CD80 and CD86 (B7-1 and B7-2, respectively) are costimulatory molecules necessary for T-cell activation. After treating the BMHC-imDCs with various concentrations of AE-PS (25, 50 and 100 $\mu\text{g/mL}$) for 48 h, a dose-dependent increase in the positive cell ratio and the mean fluorescence intensity for the expression of MHC class II molecules was observed when the cells were treated with AE-PS (Fig. 3A). When compared to the untreated control, treatment with 100 $\mu\text{g/mL}$ AE-PS increased the positive cell ratio by 41% and the mean fluorescence intensity by 28%. Similar results were obtained for CD80/86 expression. Treatment with 100 $\mu\text{g/mL}$ AE-PS increased the positive cell ratio by 42% and the mean fluorescence intensity by 26% compared to the untreated control (Fig. 3B). The increased MHC class II and CD80/86 expression levels provided further evidence that AE-PS promotes DC maturation.

3.4. Dendritic cells have reduced endocytosis after AE-PS treatment

Similar to macrophages, dendritic cells have an important role in antigen presentation by endocytosing, processing and presenting the antigens to activate the adaptive immune system (Bhardwaj 2003). According to previous studies, immature DCs have higher endocytosis and processing activities than mature DCs, and mature DCs have lower endocytosis and higher antigen-presenting activities than immature DCs (Keller, 2001). Thus, we examined if AE-PS affected the level of endocytosis in the BMHC-

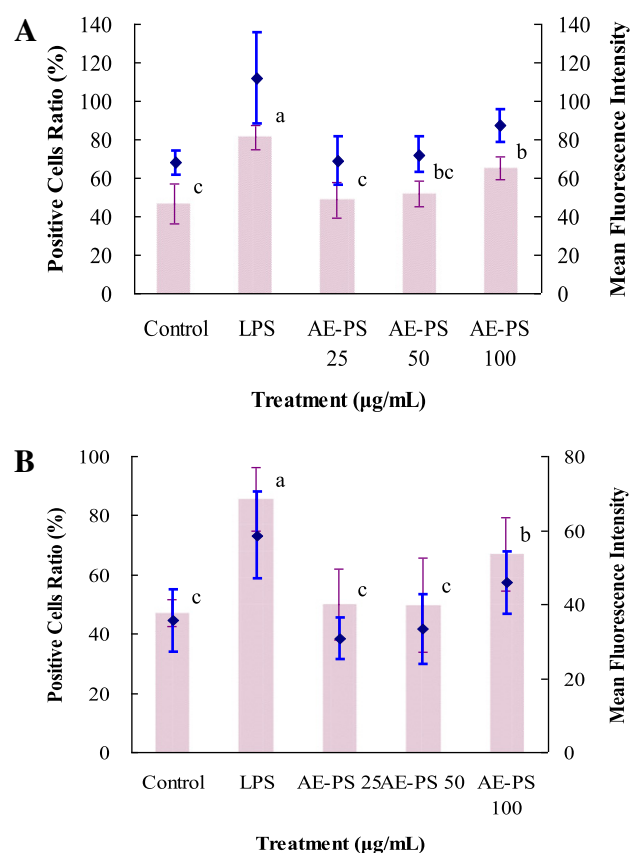


Fig. 3. Expression of (A) MHC class II and (B) CD80/86 molecules on the surface of BMHC-imDCs stimulated with AE-PS (20, 50 and 100 $\mu\text{g/mL}$) or LPS (1 $\mu\text{g/mL}$) for 48 h analysed by flow cytometry. The positive cell ratio (%) and mean fluorescence intensity are shown as columns and dots, respectively. Different letters indicate that means of positive cell ratio and fluorescence intensity are significantly different between treatment groups.

imDCs using a FITC-dextran uptake assay. For comparison purposes, untreated groups were analysed at 4 and 37 $^{\circ}\text{C}$. In terms of both positive cell ratio and mean fluorescence intensity, the AE-PS treatment reduced the endocytosis activity of DCs in a dose-dependent manner (Fig. 4). Treatment with 100 $\mu\text{g/mL}$ AE-PS decreased the positive cell ratio by 40% and the mean fluores-

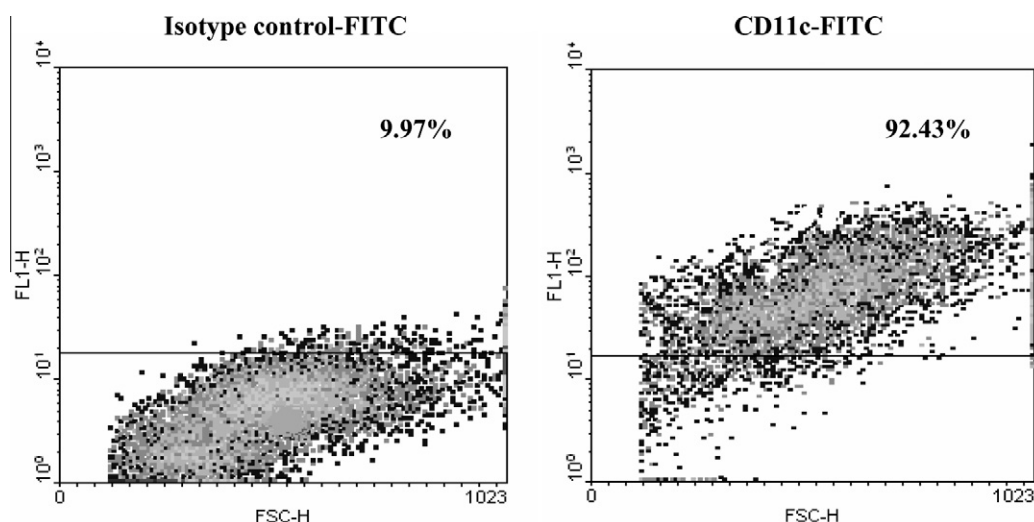


Fig. 2. Flow cytometric analysis of the CD11c cell surface marker on BMHC-imDCs after 7 days of culture. BMHC-imDCs were stained with either a FITC-conjugated isotype control or an anti-rat CD11c antibody.

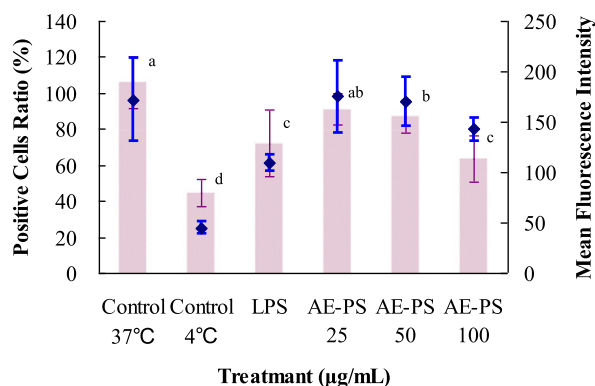


Fig. 4. Endocytosis of FITC-dextran by the BMHC-imDCs stimulated with AE-PS (20, 50 and 100 $\mu\text{g/mL}$) or LPS (1 $\mu\text{g/mL}$) for 48 h. The positive cell ratio (%) and mean fluorescence intensity are shown as columns and dots, respectively. Different letters indicate that means of positive cell ratio and fluorescence intensity are significantly different between treatment groups.

cence intensity by 17% compared to the untreated control at 37 °C. This reduction in endocytosis was comparable to that of the LPS treatment group. The reduced endocytosis activity, as shown by reduced dextran uptake, suggested that AE-PS treatment leads to the activation of DCs.

3.5. Dendritic cells have increased IL-12/IFN- γ secretion and reduced IL-10 secretion after AE-PS treatment

The cytokines secreted by DCs when they activate naïve CD4 T-cells determine the developmental path of CD4 T-cells. IL-12 and IFN- γ promote the development of CD4 T-cells into T_H1 cells, and IL-10 favours the development of T_H2 cells. As shown in Fig. 5A and B, both IL-12 and IFN- γ levels were elevated, with the most prominent increase resulting from treatment with 100 $\mu\text{g/mL}$ AE-PS. When compared to the untreated groups, the secretion of IL-12 and IFN- γ increased by 120% and 75%, respectively, when the cells were treated with 100 $\mu\text{g/mL}$ AE-PS. Treatment with 25 and 50 $\mu\text{g/mL}$ AE-PS, however, did not significantly increase the secretion levels of IL-12 and IFN- γ . Moreover, the IL-10 levels decreased as the concentration of AE-PS increased (Fig. 5C). When compared to the untreated control, treatment with 100 $\mu\text{g/mL}$ AE-PS resulted IL-10 production that was reduced by 66%. The combined result of increased IL-12/IFN- γ and reduced IL-10 production in the DCs indicated that AE-PS contributes to the T_H1 response.

4. Discussion

To the best of our knowledge, this is the first study examining the immunopotentiating effect of okra polysaccharides. This study demonstrated that AE-PS treatment promoted murine DC maturation as shown by the following characteristics: morphological changes consistent with maturation; reduced endocytosis; and increased expression of surface molecules important for antigen presentation. Furthermore, AE-PS induced the T_H1 response.

For the *in vitro* experiments, 100 $\mu\text{g/mL}$ AE-PS resulted in the best stimulation of murine DCs. Experiments performed with AE-PS concentrations above the optimal level (200, 400 and 800 $\mu\text{g/mL}$; data not shown) did not show increased DC activation, which may have been due to the saturation of surface receptors or the presence of unidentified components of the AE-PS coming into play as the concentration increased. Analysis of individual components of the hydrolysed extract will provide a clearer picture. When compared to LPS, AE-PS exerted a weaker stimulatory effect on the DCs, which may be an advantage if the AE-PS is used as an

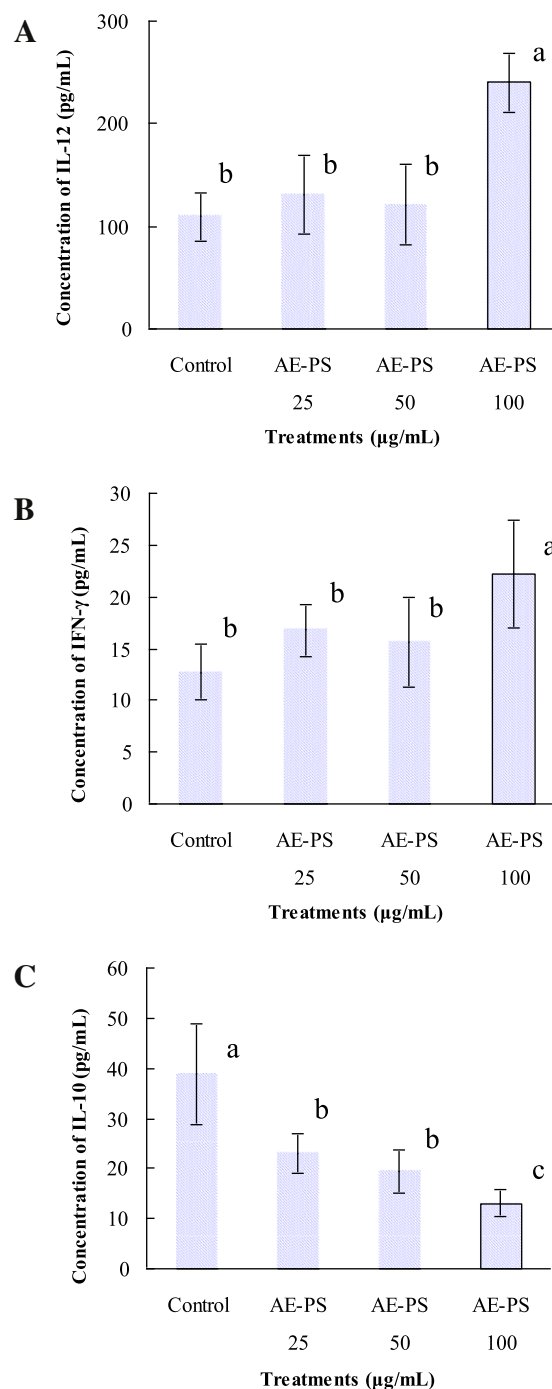


Fig. 5. Production of (A) IL-12, (B) IFN- γ and (C) IL-10 in BMHC-imDCs treated with AE-PS (20, 50 and 100 $\mu\text{g/mL}$). The cytokine levels in the supernatants were detected by ELISA. The data are shown as the mean $\pm$ SD ($n = 3$). Different letters indicate that means of IL-12, IFN- γ and IL-10 levels are significantly different between treatment groups.

adjuvant. The activation pathway of AE-PS may be distinct from the activation pathway of LPS, thereby, adding to the repertoire of adjuvants for vaccine development or tumoricidal treatments.

Although this study suggested that okra polysaccharides have an effect on DCs, the exact component responsible for this activation remains unknown. Previous studies have suggested that β -glucan has a role in immune stimulation by activating macrophages, natural killer cells, and a minor subset of B-cells and T-cells through C3 receptors (Ross & Vetvicka, 1993). However, the sugar-binding specificity of the C3 receptor may extend to include certain

polysaccharides containing mannose, N-acetyl-D-glucosamine and glucose (Thornton, Vetvicka, Pitman, Goldman, & Ross, 1996). Analysis of the aqueous extract of okra showed a β -glucan content of 0.6%. Thus, future experiments may involve the exclusion of β -glucan from the extract to determine if it is principally responsible for immune activation. Moreover, polysaccharides may not be the only important component in the okra extract. In fungal extracts, for example, triterpenes also have an immunomodulatory role (Dudhgaonkar, Thyagarajan, & Sliva, 2009).

The present study demonstrated that AE-PS stimulated DCs to produce IL-12 and IFN- γ , leading to a T helper type 1 response and suggesting that okra polysaccharides may be applied to treat allergy-related diseases. Studies have shown that *Antrodia camphorata* polysaccharides can alleviate allergen-induced T helper type 2 responses in a mouse model, thereby, relieving asthma symptoms (Geethangili & Tzeng, 2011; Liu et al., 2010). The reduced production of IL-10 in AE-PS-treated DCs indicated that okra polysaccharides may also be useful to relieve asthma symptoms.

5. Conclusion

The hydrolysed okra extracts exhibited promising immunopotentiating effects. AE-PS activated BHMC-imDCs and upregulated MHC class II and CD80/86 molecules. AE-PS-treated dendritic cells exhibited increased IL-12/IFN- γ secretion and reduced IL-10 secretion, indicating a T_H1 immune response. Future investigations exploring the active ingredients and delineating the signalling pathways involved in the AE-PS effect will aid in the development of AE-PS as a component of therapeutic treatments.

Acknowledgements

The authors would like to thank all of the colleagues and students who contributed to this study. The scientific and editing input of Dr. Li-Ting Cheng was also greatly appreciated. The authors would also like to thank the anonymous reviewers and an editor for their comments.

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