Shang-Tse Ho, Yu-Tang Tung, Yu-Jung Wu, Chi-Chen Lin* and Jyh-Horng Wu*

Immune-regulatory activity of methanolic extract of *Acacia confusa* heartwood and melanoxetin isolated from the extract

Abstract: The antioxidant, anti-inflammatory, antivirus, uric acid decreasing, and hepatoprotective activities of *Acacia confusa* extracts were demonstrated in previous studies. However, there is no scientific evidence concerning the immune-regulatory activity of the heartwood extract of *A. confusa*. In this study, the effect of a methanolic heartwood extract (MHE) from *A. confusa* on dendritic cell (DC) activation and function was examined. *A. confusa* MHE significantly reduced the production of pro-inflammatory cytokine interleukin-6 (IL-6) in lipopolysaccharide (LPS)-stimulated DCs, and the effective concentration (25 μg ml^{-1}) of *A. confusa* MHE did not affect cell viability. Additionally, the bioactive phytochemical from *A. confusa* MHE, melanoxetin, was isolated and purified by HPLC. This substance inhibited the production of pro-inflammatory cytokines (tumor necrosis factor-α, IL-6, and IL-12) in LPS-stimulated DC at a concentration of 12.5 μM. Moreover, the expression levels of co-stimulatory molecules such as CD40, CD80, and CD86 also remarkably decreased after treatment with melanoxetin at the same dose. These findings indicate that *A. confusa* MHE and melanoxetin have excellent immune-suppressive activity and may be potential candidates for further development of natural health supplements.

Keywords: *Acacia confusa*, dendritic cell, heartwood, immune-regulatory activity, melanoxetin

***Corresponding authors: Chi-Chen Lin,** Institute of Biomedical Science, National Chung Hsing University, Taichung 402, Taiwan, Tel.: +886 4 22840345, Fax: +886 4 22851308, e-mail: lincc@nchu.edu.tw; and **Jyh-Horng Wu**, Department of Forestry, National Chung Hsing University, Taichung 402, Taiwan, Tel.: +886 4 22840345, Fax: +886 4 22851308, e-mail: eric@nchu.edu.tw

Shang-Tse Ho: Department of Forestry, National Chung Hsing University, Taichung 402, Taiwan

Yu-Tang Tung: Department of Life Sciences, National Chung Hsing University, Taichung 402, Taiwan

Yu-Jung Wu: Institute of Biomedical Science, National Chung Hsing University, Taichung 402, Taiwan

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Introduction

The extracts of many woody plants are traditionally used as biocides (Anttila et al. 2013; Chien et al. 2013; Kadir et al. 2014). Recently, natural plant resources have received attention for their pharmacological properties, and the immune-regulatory components from native resources have also been widely screened (Horstmann et al. 2007; Makino et al. 2011; Miller et al. 2011; Telysheva et al. 2011; Uto et al. 2011; Hua et al. 2013; Rao et al. 2013). *Acacia confusa* Merr. is one of the most common hardwood species widely distributed in Taiwan, especially in the hills and lowlands. The leaf extract from *A. confusa* is a traditional folk medicine for treating wound and as a treatment for blood stasis (Wu et al. 2008a; Tung et al. 2009a). Increasing evidence indicates that extracts from *A. confusa* and derived phytochemicals are antioxidant, anti-inflammatory, antivirus, uric acid decreasing, xanthine oxidase inhibitory, and hepatoprotective (Wu et al. 2005, 2008a,b; Tung et al. 2007, 2009a,b,c, 2010, 2011; Tung and Chang 2010a,b; Lee et al. 2011). According to the quoted studies, phenolic compounds such as phenolic acids and flavonoids are the major bioactive phytochemicals in *A. confusa* extracts. In addition, melanoxetin, one of the major substances in the extract, shows xanthine oxidase inhibition (Tung and Chang 2010a), reduces serum uric acid levels in oxonate-induced mice (Tung et al. 2010), and is prone to inflammatory mediator production (Wu et al. 2008b).

The flavonoids are a subclass of polyphenolic compounds with a lower molecular weight than usual in many plants. Some *Acacia* species have been reported to produce abundant flavonoids (Clark-Lewis and Porter 1972; Thieme and Khogali 1975; Lee and Chou 2000; Lee et al. 2000; Pietarinen et al. 2005; Wu et al. 2005; Dongmo et al. 2007). Various bioactivities for flavonoids have been

verified, including immune-regulatory, anti-carcinogenic, cardiovascular protective, anti-inflammatory, antioxidant, and bone-protective effects, as well as amelioration of metabolic syndrome-related diseases (Wu et al. 2005; Benavente-García and Castillo 2008; Huang et al. 2010; Sharan et al. 2011; Galleano et al. 2012; Welch et al. 2012; Rosales-Castro et al. 2012).

Dendritic cells (DCs) are antigen-presenting cells, and the major function of DCs is to initiate and mediate the immune response in the innate and adaptive immune system (Banchereau and Steinman 1998; Guermonprez et al. 2002). Owing to the importance of their function in the immune system, DCs served as an experimental platform for evaluating the regulatory effects of test materials on the immune response (Huang et al. 2010; Lin et al. 2011a). To the best of our knowledge, there is no prior research on the immune-regulatory activities of *A. confusa* extracts and their derived phytochemicals in the context of the DC platform. The aim of this study was to investigate the regulatory effects of *A. confusa* extracts on the immune response, with the DC platform serving as a model. In the present study, the effect of *A. confusa* heartwood extract and its major phytochemical, melanoxetin, on DC activation and function was examined.

Materials and methods

Cell counting kit-8 (CCK-8), lipopolysaccharide (LPS), dimethyl sulfoxide, and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). RPMI 1640 medium, fetal bovine serum (FBS), and other cell culture reagents were obtained from Gibco (Grand Island, NY, USA). CD11c, CD40, CD80, and CD86 mouse antibodies were purchased from eBioscience (San Diego, CA, USA). All of the other chemicals and solvents used in this research were of analytical grade.

Heartwood of *A. confusa* was kindly provided by Mr. Ming-Jay Chung, assistant researcher of the experimental forest of National Taiwan University. The species was identified by Dr. Yen-Hsueh Tseng, and the voucher specimen (Lu 0069) was deposited at the herbarium of the Department of Forestry, National Chung Hsing University. The heartwood was chopped into small pieces and extracted by soaking in methanol (MeOH) for one week at room temperature (25°C) twice. The methanolic heartwood extract (MHE) was decanted, filtered under a vacuum, concentrated in a rotator evaporator, and then lyophilized. The MHE was stored in an airtight container at -40°C until further use.

The bioactive phytochemical from *A. confusa* MHE was separated and purified by semi-preparative HPLC on a Jasco PU-2080 instrument (Tokyo, Japan) with a 250 mm×10.0 mm i.d. and a 5 μm Supelco RP-amide column (Bellefonte, PA, USA). Before HPLC isolation, the MHE was purified with a solid-phase extraction (SPE) step to remove non-polar substances. In brief, the MHE was dissolved in MeOH and loaded onto a Strata C-18E SPE cartridge (Phenomenex, Torrance, CA, USA). The purified MHE was eluted with MeOH, concentrated in a rotary evaporator, and then re-dissolved in MeOH/water for HPLC separation. The mobile phase consisted of solvent A (100% MeOH) and solvent B (ultrapure water). The elution conditions were 0–24 min of 4%–100% A (linear gradient) at a flow rate of 4 ml min^{-1}, detected using 370 nm Jasco MD-2010 photodiode array detector (Tokyo, Japan). The chemical structure of melanoxetin was identified by NMR spectroscopy using Bruker Avance 500 MHz FTNMR spectrometer (Rheinstetten, Germany). Chemical shifts are presented in δ (ppm) relative to the solvent CD_3OD (δ_H 3.31, δ_C 49.1) as an internal standard. All NMR spectral data were consistent with those of the literature (Wu et al. 2005).

C57BL/6 mice were purchased from National Cheng Kung University Laboratory Animal Center (Tainan, Taiwan). Mice were given a standard laboratory diet and distilled water *ad libitum*. In addition, they were maintained in a 12 h light/dark cycle at 22±2°C. This study was conducted according to the institutional guidelines of and approved by the Institutional Animal Care and Utilization Committee of National Chung Hsing University, Taiwan. DCs derived from mouse bone marrow were generated following the method of Chu and Lowell (2005) with slight modifications. Briefly, bone marrow cells were isolated from the femurs and tibias of mouse hind legs and seeded on 6-well culture plates with 4 ml of RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 10 ng ml^{-1} recombinant mouse granulocyte-macrophage colony-stimulating factor (GM-CSF), and 10 ng ml^{-1} recombinant mouse interleukin-4 (IL-4). On Days 3 and 5, 2 ml of fresh medium containing 10 ng ml^{-1} GM-CSF and 10 ng ml^{-1} IL-4 was added to the 6-well culture plates. DCs cultured for seven days were harvested and used for all of the experiments in this study.

Cytokine production was measured using commercial kits (PeproTech, Rocky Hill, NJ, USA; eBioscience, San Diego, CA, USA) as described previously (Huang et al. 2010). DCs were pretreated with various concentrations of *A. confusa* MHE (final concentrations were 25, 50, and 100 μg ml^{-1}) or melanoxetin (final concentrations were 6.25, 12.5, 25, and 50 μM) for 1 h, then stimulated with LPS (100 ng ml^{-1}) for 6 h or 24 h to assess the production of tumor necrosis factor-α (TNF-α) or interleukins (IL-6 and IL-12), respectively. After incubation, the culture medium from each well was collected and centrifuged for 5 min (1250 rpm). The cytokine levels in the supernatant were quantified using an ELISA reader (Labsystems Multiskan MS, Helsinki, Finland). Three replicates were made for each test sample.

DCs were seeded into a 96-well plate at a density of 2×10^5 cells/well and incubated overnight, then treated with various concentrations of *A. confusa* MHE (25, 50, and 100 μg ml^{-1}) or melanoxetin (6.25, 12.5, 25, and 50 μM) for 24 h. After incubation, 10 μl of CCK-8 solution was added to each well and incubated in a CO_2 incubator for 3 h. Cell viability was quantified by measuring the absorbance at 450 nm. Three replicates were made for each test sample.

DCs were seeded into a 6-well plate at a density of 2×10^6 cells/well and incubated overnight, then pretreated with 12.5 μM or 25 μM melanoxetin for 1 h following stimulation with 100 ng ml^{-1} LPS for 24 h. Subsequently, the cells were harvested and washed with 0.1% BSA in phosphate buffered saline twice, and then stained for CD11c + CD40, CD11c + CD80, or CD11c + CD86. The expression levels of each molecule were analyzed by flow cytometry (BD Accuri™ C5, BD BioSciences, Franklin Lakes, NJ, USA). Three replicates were made for each test sample.

All results are expressed as the mean±SD (n=3). Statistical analysis was performed with one-way ANOVA and Scheffe's post-hoc test. The results with $P<0.05$ were considered to be statistically significant.

Results and discussion

Inhibition of the production of pro-inflammatory cytokines in LPS-stimulated DC

It is well known that pro-inflammatory cytokines such as TNF-α and IL-6 are the hallmarks of DC activation (Trucci et al. 2013; Dalod et al. 2014). In the present study, DCs expressed pro-inflammatory cytokines after LPS (100 ng ml^{-1}) stimulation. Compared with the vehicle control group, the TNF-α production in LPS-stimulated DC (2.3 ng ml^{-1}) was 10-fold higher than that of the vehicle control group (0.2 ng ml^{-1}) (Figure 1a). In addition, the TNF-α production of LPS-stimulated DCs was decreased after treatment with high concentrations of *A. confusa* MHE (100 μg ml^{-1}), indicating that *A. confusa* MHE might potentially inhibit the TNF-α production of DCs. However, the lower dosage of *A. confusa* MHE treatment group had no significant inhibitory effect on TNF-α production. Furthermore, because IL-6 is also an important cytokine during DC activation, the effect of *A. confusa* MHE on IL-6 production was determined. As shown in Figure 1b, *A. confusa* MHE significantly reduced the production of IL-6 levels in LPS-stimulated DCs in a dose-dependent manner. Treatment with 25 μg ml^{-1} of *A. confusa* MHE remarkably decreased the IL-6 expression level by 20.6%. According to these results, *A. confusa* MHE decreased the production of pro-inflammatory cytokines and inhibited LPS-induced DC activation. To ensure that the inhibitory effect of *A. confusa* MHE on DC activation was not due to the cytotoxic effects of *A. confusa* MHE on the cells, the cytotoxicity of *A. confusa* MHE was also assessed using the CCK-8 cytotoxicity assay in this study. As shown in Figure 2, high concentrations (50 and 100 μg ml^{-1}) of *A. confusa* MHE exhibited cytotoxic effects on DCs. However, there was no significant cytotoxicity at 25 μg ml^{-1} of *A. confusa* MHE, in which the cell viability remained at 89.4%. Accordingly, it was demonstrated for the first time that *A. confusa* MHE has an immune-regulatory activity on LPS-stimulated DCs and implied that the MHE contains immune-suppressive phytochemicals. Thus, *A. confusa* MHE might be a potential inhibitor of the DC-related immune response and attenuate harmful immune activities in the human body.

Isolation and identification of bioactive phytochemicals

As demonstrated above, *A. confusa* MHE and derived phytochemicals might be suitable candidates for the development of natural medicine. Additionally, the immune-regulatory effects of flavonoids have been well verified and validated in previous studies (Huang et al. 2010; Lin et al. 2011b). According to Wu et al. (2005, 2008b), flavonoids are one of the major components of *A. confusa* MHE, and this substance class is probably responsible for the observed immune-regulatory effect.

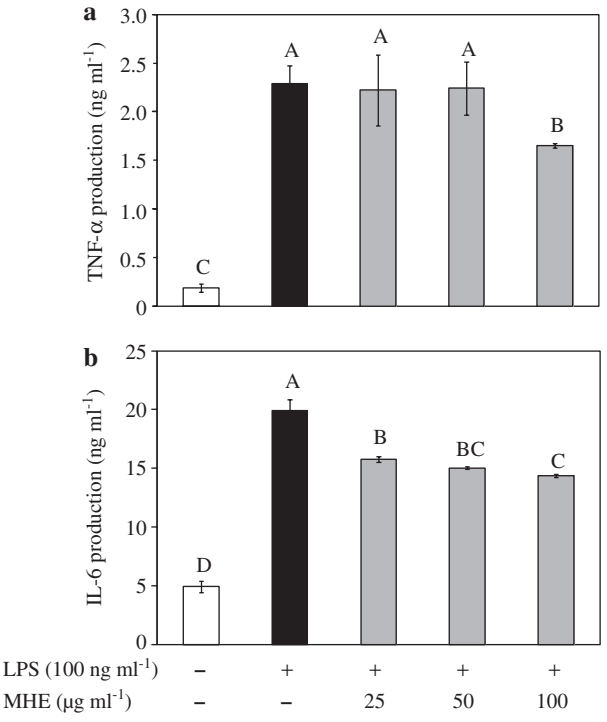

Figure 1 Inhibitory effect of *Acacia confusa* methanolic heartwood extract on the production of the pro-inflammatory cytokines TNF-α (a) and IL-6 (b). Values are means±SD (n=3). Bars with different capital letters indicate significant differences at P<0.05.

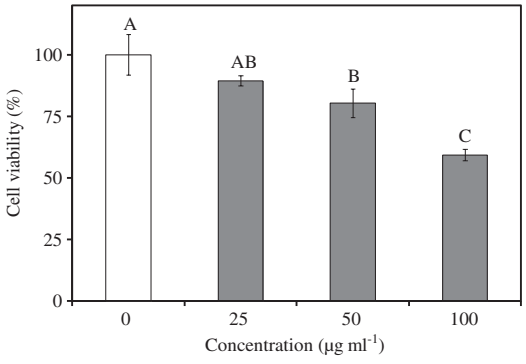

Figure 2 Effect of *Acacia confusa* methanolic heartwood extract on the viability of dendritic cells. Values are means±SD (n=3). Bars with different capital letters indicate significant differences at P<0.05.

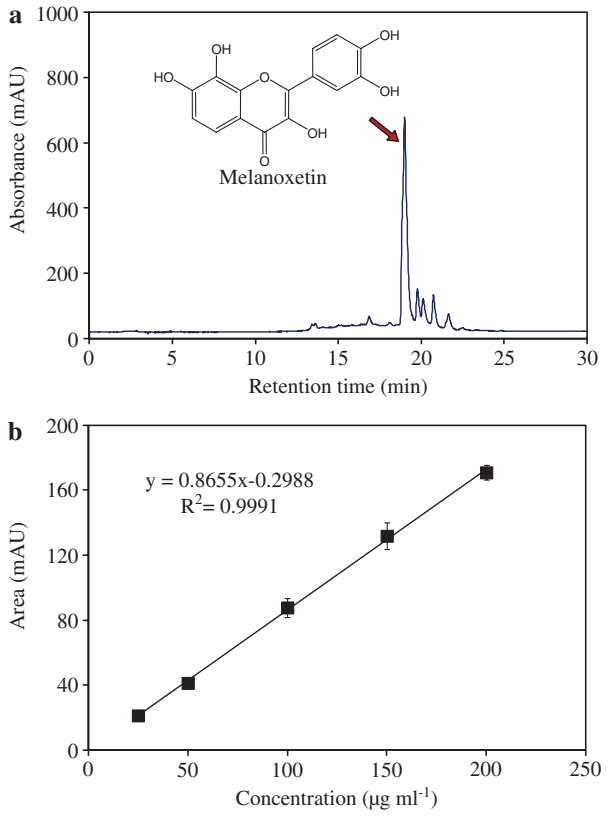

Figure 3 (a) HPLC chromatography profile of *Acacia confusa* methanolic heartwood extract (detection at 370 nm). (b) The absorption peak area at 370 nm is plotted against the concentration of melanoxetin. Three replicates were made for each test sample.

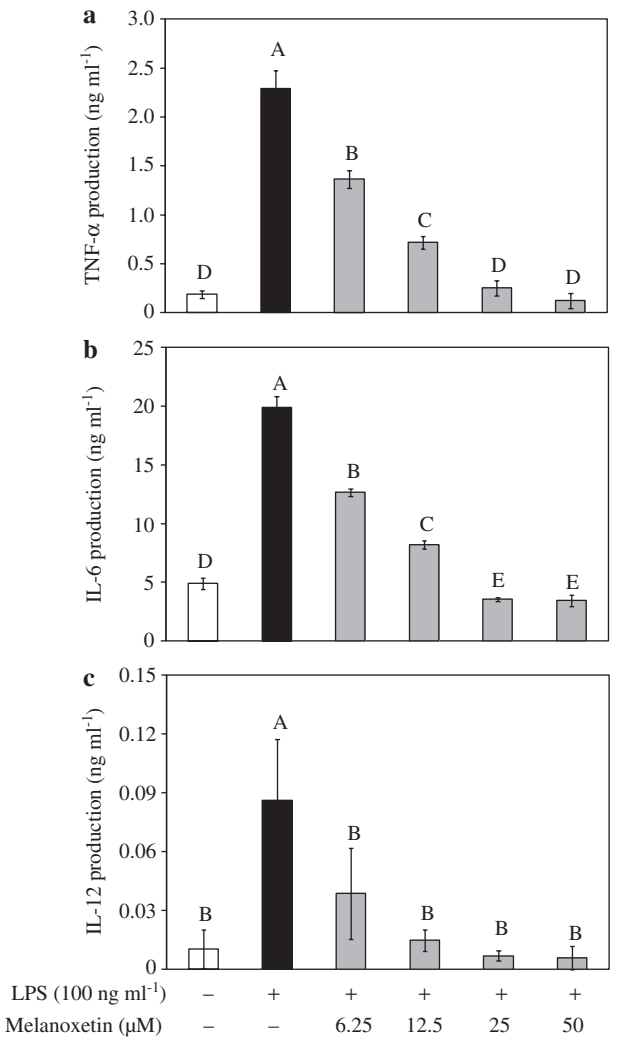

Figure 4 Inhibitory effect of melanoxetin on the production of the pro-inflammatory cytokines TNF-α (a), IL-6 (b), and IL-12 (c). Values are means±SD (n=3). Bars with different capital letters indicate significant differences at $P<0.05$.

The phytochemicals from *A. confusa* MHE were further isolated and identified in a semi-prepared HPLC system and the NMR spectrum, respectively. As shown in Figure 3a, melanoxetin was the principal phytochemical in the *A. confusa* MHE and its content was determined to be 0.14±0.004 g per 100 g of air-dried heartwood. The calibration curve (R^2=0.999) is shown in Figure 3b.

Inhibition effect of melanoxetin

To evaluate the effect of the isolated melanoxetin, tests were performed concerning its inhibitory effect on pro-inflammatory cytokine production by means of the LPS-stimulated DC assay. The result of TNF-α production is shown in Figure 4a. After pretreatment with various concentrations (6.25, 12.5, 25, and 50 μM) of melanoxetin, the TNF-α level in LPS-stimulated DC decreased in a dose-dependent manner, revealing the positive effect of melanoxetin in this context. In addition, given that IL-6 and IL-12 are also important cytokines involved in the DC activation process, the effects of melanoxetin on the production of these cytokines were further determined using the DC platform. As shown in Figure 4b, after pretreatment with 6.25, 12.5, 25, or 50 μM of melanoxetin, IL-6 levels decreased from 19.9 ng ml^{-1} to 12.6, 8.2, 3.6, and 3.4 ng ml^{-1}, respectively, demonstrating a significant inhibitory effect on IL-6 production. Compared with the IL-6 levels in the LPS-stimulated group, the IL-6 production of the 25 μM melanoxetin treatment groups was 5-fold lower than that of the LPS-stimulated group. In addition, the IL-12 production in LPS-stimulated DCs was consistent with the IL-6 production assay; the cytokine levels were attenuated by treatment with melanoxetin in a dose-dependent manner. These results indicate that one of the possible mechanisms

accounting for this observation might be by suppression of pro-inflammatory cytokine production. Phloretin, a naturally occurring dihydrochalcone phytochemical that has been reported to have suppressive effect on the production of pro-inflammatory cytokines in LPS-stimulated DCs (Lin et al. 2014), also had an inhibitory effect on TNF-α, IL-6, and IL-12 production at a concentration of 25 μM, which was approximately 50%, 40%, and 25%, respectively. In contrast, the same concentration of melanoxetin inhibited 89%, 82%, and 92% of TNF-α, IL-6, and IL-12 production, respectively. Quercetin is another well-known immune-suppressive compound, and its inhibitory effect at a concentration of 25 μM on TNF-α, IL-6, and IL-12 production was approximately 60%, 55%, and 70%, respectively (Huang et al. 2010). This comparison demonstrates that melanoxetin is an effective inhibitor of pro-inflammatory cytokine production.

Cytotoxic effect of melanoxetin

To exclude the possible effects of cytotoxicity, the cell viability of DCs after treatment with melanoxetin was determined by the CCK-8 assay. As shown in Figure 5, the cell viability of DC after treatment with 6.25–25 μM of melanoxetin was higher than 85%. However, melanoxetin showed relatively higher cytotoxicity with a cell viability of 80.8% at a concentration of 50 μM. Accordingly, melanoxetin is an active immune-regulator inhibiting the production of pro-inflammatory cytokines and did not affect DC viability at concentrations below 25 μM. The cytotoxic effect of flavonoids on DCs was previously reported. Xuan et al. (2010) found that xanthohumol, a flavonoid constituent of beer, induced apoptosis and activation of caspase family proteins in mouse bone marrow-derived DCs. Additionally, Huang et al. (2010) reported the immune-suppressive effect of quercetin, but they also found that higher concentration (100 μM) of quercetin significantly induced apoptosis in DC. These reports indicate that flavonoids induce apoptosis at a certain concentration, and the cytotoxicity of high concentration of melanoxetin might contribute to its capacity to initiate apoptosis in DCs.

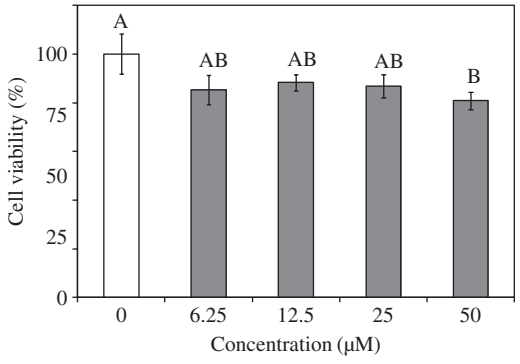

Figure 5 Effect of melanoxetin on the viability of dendritic cells. Values are means±SD (n=3). Bars with different capital letters indicate significant differences at P<0.05.

Melanoxetin impairs LPS-stimulated DC maturation

DCs are professional antigen-presenting cells and regulate crucial steps in the immune system (Johnson and Ohashi 2013). When DCs mature, their specific regulatory effects on the immune response are initiated. In addition, the expression of the co-stimulatory molecules CD40, CD80, and CD86 increase in mature DCs. Therefore, the inhibitory effect of melanoxetin on co-stimulatory molecule expression in the LPS-stimulated DC model was determined in this study. As shown in Figure 6, the result of flow cytometry analysis indicates that the mean fluorescence intensity (MFI) of CD40, CD80, and CD86 on LPS-stimulated DC was enhanced from initial levels of 12.6, 61.2, and 52.8 to 24.5, 115.0, and 102.9, respectively. However, after treatment with 12.5 μM melanoxetin, the MFI of CD40, CD80, and CD86 decreased by 45.7%, 42.3%, and 18.2%, respectively. By increasing the concentration of melanoxetin to 25 μM, the MFI of CD40, CD80, and CD86 further decreased by 55.1%, 58.8%, and 55.4%, respectively, suggesting that LPS-induced DC maturation could be attenuated by melanoxetin in a dose-dependent manner. These findings show that melanoxetin has the potential to regulate the immune system.

Antioxidant activity is a possible mechanism causing the immune-suppressive effect of melanoxetin. Reactive oxygen species (ROS) are a signaling molecule involved in many cellular events. Recently, many studies have reported that ROS played a critical role in DC activation by influencing the maturation process, cytokine production and release, and the antigen-presenting activity of both human- and mouse-derived DCs (Karlsson et al. 2007; Sheng et al. 2010; Lin et al. 2014). In contrast, another study showed that treatment with the antioxidant *N*-acetyl-L-cysteine reduced the immune responses and exhibited inhibitory action on DCs (Verhasselt et al. 1999). It is well known that melanoxetin is an excellent ROS scavenger (Wu et al. 2005). Accordingly, the immune-regulatory activity of melanoxetin on DC maturation might partially be due to ROS scavenging activity and deserves further investigation.

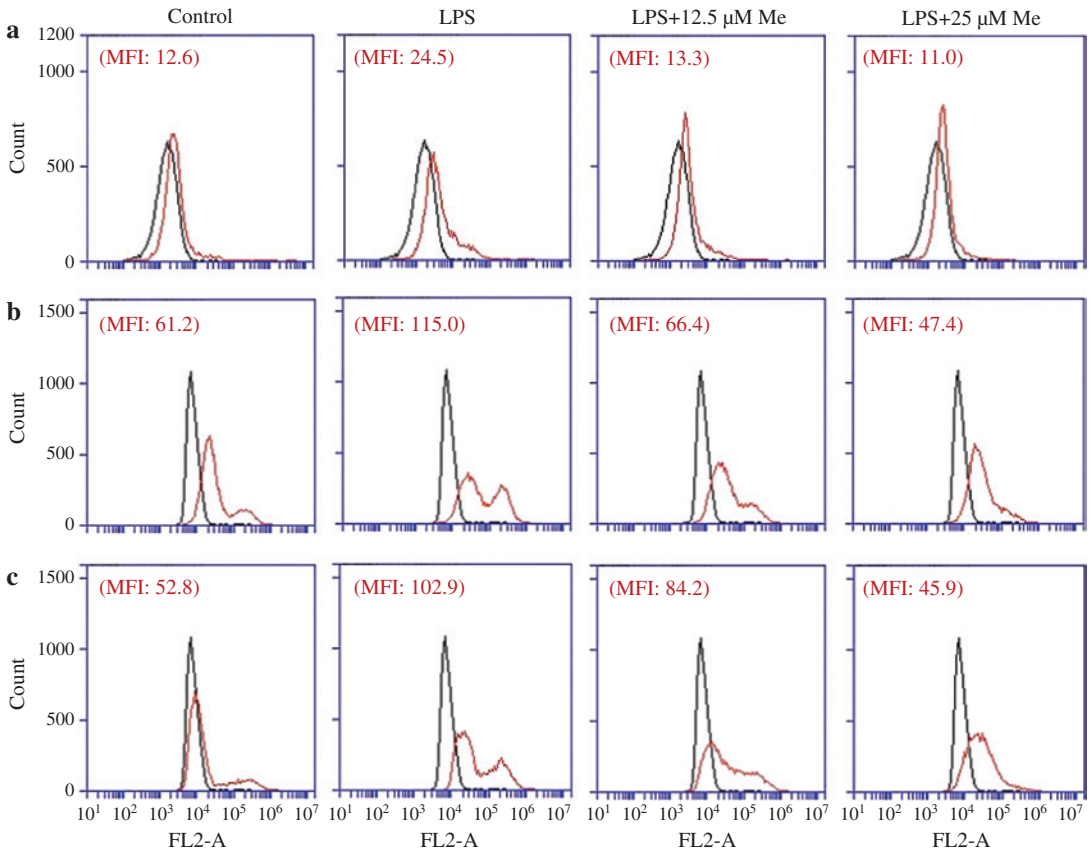

Figure 6 Inhibitory effect of melanoxetin on lipopolysaccharide (LPS)-induced dendritic cell (DC) maturation. DCs were treated with vehicle (Control), 100 ng ml^{-1} LPS + vehicle (LPS), 100 ng ml^{-1} LPS+12.5 μM melanoxetin (LPS+12.5 μM Me), or 100 ng ml^{-1} LPS+25 μM melanoxetin (LPS+25 μM Me) for 24 h. The expression of CD 40 (a), CD80 (b), and CD86 (c) were determined by flow cytometry. The mean fluorescence intensity of each group is indicated. Black and red curved lines represent unstained and stained cells, respectively.

Conclusions

Acacia confusa MHE was examined for its immune-regulatory activities. MHE significantly reduced the production of the pro-inflammatory cytokine IL-6 in LPS-stimulated DCs. In addition, melanoxetin was the major bioactive flavonoid from *A. confusa* MHE and inhibited the production of pro-inflammatory cytokines (TNF-α, IL-6, and IL-12) in LPS-stimulated DCs. This substance down-regulated the expression of the co-stimulatory molecules CD40, CD80, and CD86, and exhibited no cytotoxicity on DCs below concentrations of 25 μM. Taken together, *A. confusa* MHE and the isolated melanoxetin showed excellent immune-suppressive activity in the DC platform, demonstrating their potential for treating diseases caused by immune overactivation. In general, the immunopharmacology of *A. confusa* MHE and melanoxetin might be medically useful for enhancing immune regulation. This is an example of how to gain value-added products from the forest and wood products by the extraction of compounds for natural medicine.

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