Research Article

Immunosuppressive Effects of A-Type Procyanidin Oligomers from Cinnamomum tamala

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Received 10 July 2014; Revised 13 September 2014; Accepted 16 September 2014; Published 30 October 2014

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Cinnamon barks extracts have been reported to regulate immune function; however, the component(s) in cinnamon barks responsible for this effect is/are not yet clear. The aim of this study is to find out the possible component(s) that can be used as therapeutic agents for immune-related diseases from cinnamon bark. In this study, the immunosuppressive effects of fraction (named CT-F) and five procyanidin oligomers compounds, cinnamtannin B1, cinnamtannin D1 (CTD-1), parameritannin A1, procyanidin B2, and procyanidin C1, from Cinnamomum tamala or Cinnamomum cassia bark were examined on splenocytes proliferation model induced by ConA or LPS. Then, the effects of activated compound CTD-1 on cytokine production and 2,4-dinitrofluorobenzene (DNFB) induced delayed-type hypersensitivity (DTH) response were detected to evaluate the immunosuppressive activity of CTD-1. It was found that CT-F and CTD-1 significantly inhibited the splenocyte proliferation induced by ConA or LPS. CTD-1 dose-dependently reduced the level of IFN-γ and IL-2 and intensively suppressed DNFB-induced DTH responses. These findings suggest that the immunosuppressive activities of cinnamon bark are in part due to procyanidin oligomers. CTD-1 may be a potential therapeutic agent for immune-related diseases.

1. Introduction

Cinnamon is well known as a spice that is mainly distributed in Asia, South America, and the Caribbean [1]. Cinnamomum cassia, the most popular type of cinnamon, has been used for thousands of years in traditional Chinese medicine (TCM) for treating various disorders such as chronic gastric symptoms, circulation disturbances, arthritis, and common cold. Other species, such as C. japonica, C. burmannii, and C. chinii, are used as cinnamon in some parts of China. The use of cinnamon barks to treat rheumatoid arthritis (RA), a chronic inflammatory autoimmune disease, was described in an ancient Chinese medical book “Tai Ping Sheng Hui Fang,” owing to its function of warming yang for dispelling cold. Many modern clinical experiences have also reported that cinnamon bark powder (or the prescriptions which contain cinnamon) is useful in treating RA although the key component(s) of this action is/are still unknown [2–4].

Cinnamon bark is reported as a good antiarthritic agent because of its anti-inflammation, pain relieving, and immune regulation function [5]. The anti-inflammatory activity of cinnamon species has been attributed to a series of procyanidin oligomers found in its barks [6]. Recently, literatures report that the water-soluble cinnamon bark extract had potential effects in regulating immune function in vitro and in preventing and treating inflammation-related diseases [7–9]. These results suggested that the water-soluble components in cinnamon, such as procyanidin oligomers, may play an important role in the therapeutic action of RA; however, the fact needs more studies to confirm.

Procyanidins oligomers are widely distributed in plants. They are mainly classified as A-type and B-type based on the linkage between the successive flavan-3-ol units. Previous studies found that cinnamon species contained both the A-type and B-type procyanidin oligomers [10, 11]. Recently, our studies found that C. cassia contained mainly B-type procyanidin dioligomer and trioligomer, whereas C. tamala, mainly used in Yunnan Province of China, contains mainly A-type procyanidin trioligomers. The different types of procyanidin oligomers may have different effects just as they have diverse antidiabetes mechanisms [12, 13].
To determine whether procyanidin oligomers in cinnamon species have immunosuppressive effects and whether the different types have varying effects on immunosuppression, five procyanidin oligomers isolated from cinnamon barks including three A-type procyanidin oligomers which are main constituents in C. tamala bark and two B-type procyanidin oligomers which are main constituents in C. cassia bark were evaluated for inhibitory effects of ConA-activity induced splenocyte proliferation. Furthermore, the anti-RA CT-D-1 among the five procyanidin oligomers was studied.

2. Materials and Methods

2.1. Materials and Reagents. C. cassia and C. tamala were collected from Guangxi and Yunnan Provinces, respectively. The samples were botanically authenticated by Professor Guanyun Gu at the School of Pharmacy, Fudan University. The voucher specimens, numbers RG006 and RG007, were deposited at the Herbarium of the Department of TCM Chemistry, School of Pharmacy of Shanghai University of Traditional Chinese Medicine (Shanghai, China).

RPMI 1640 powder was purchased from Gibco. ConA and LPS were purchased from Sigma. [3H]-Thymidine (1 mCi/mL) was purchased from the Shanghai Institute of Atomic Energy. ELISA kits for IL-2 and IFN-γ were purchased from BD PharMingen (San Diego, CA, USA). 2,4-Dinitrofluorobenzene (DNFB) and other reagents were of analytical grade.

2.2. Experimental Animals. Female BALB/c mice, aged 6 weeks to 8 weeks, were purchased from the Shanghai Laboratory Animal Center, Chinese Academy of Sciences. The animals were housed in a specific pathogen-free environment (22 ± 1°C, RH 55 ± 5%, and 12 h light-to-dark cycle) at least 6 days before use. All procedures were carried out in accordance with the Chinese legislation on the use and care of laboratory animals and were approved by the respective university committees for animal experiments (Permission number: 2013018).

2.3. Extraction and Isolation. The preparation of procyanidin oligomers enriched extracts from two cinnamon species, C. tamala and C. cassia bark, was described in our previous paper [13]. Then the compounds were systematically isolated by using column chromatography and HPLC semipreparation. Briefly, 25 g of EtOAc fraction of C. tamala was dissolved in a small amount of MeOH, diluted with water, chromatographed on an open macroporous resin DM130 column (6.0 cm × 50.0 cm), and eluted with gradient of MeOH-H2O (0%, 10%, 40%, and 70% of MeOH) to yield 40% MeOH fraction 12.6 g, which was labeled CT-F. CT-F was dissolved in 10% aqueous acetonitrile, subjected to ODS-A-HG C18 column (4.0 cm × 35.0 cm), and eluted with 10% acetonitrile to yield six fractions (A1 to A6). A4 and A5 fractions were applied to Toyopearl HW-40F column chromatography (3.0 cm × 40.0 cm, eluted with 70% MeOH) to yield freeze-dried powder compounds 1 (300 mg) and 2 (230 mg), respectively. The A2 fraction was separately applied to preparative HPLC (12% acetonitrile) to produce freeze-dried powder compound 3 (15 mg).

The C. cassia bark was processed by performing the same steps as those used for CT-F fraction to yield 40% MeOH fraction 2.7 g. Then, the fraction was subjected to Sephadex-LH20 column (3.5 cm × 50.0 cm, isocratically eluted with 50% MeOH) and, thereafter, ODS-A-HG C18 column chromatography (2.5 cm × 30.0 cm, isocratically eluted with 10% acetonitrile) to yield freeze-dried powder compounds 4 (100 mg) and 5 (40 mg).

2.4. Characterization of A-Type Procyanidin Oligomers (TAPOs) from CT-F. The obtained fraction (CT-F) and compounds (1–5) were characterized by high performance liquid chromatography (HPLC). HPLC analyses were performed on Waters ACQUITY UPLC H-class instrument equipped with a sample manager-FTN, quaternary solvent manager, column heater, photodiode array (PDA) detector, and Empower-Station for data collection and manipulation. An Agilent Extent-C18 column (5 μm; 4.6 × 250 mm) was used in this experiment. The samples were analyzed using a solvent system with 0.1% acetic acid (A) and acetonitrile (B) under the following conditions: linear gradients from 12% to 18% B for 20 min, 45% solvent B for 25 min, 70% solvent B for 30 min, and 90% B for 35 min at a flow rate of 1 mL/min at 280 nm.

2.5. Cell Viability. Cell viability was assessed by performing MTT assay [14]. Briefly, splenocytes (4 × 10⁵ cells/well) were cultured in triplicate in the absence or presence of CT-F or five procyanidin oligomers in a 96-well plate for 48 h at 37°C in a 5% CO₂ atmosphere for 48 h. The incubation mixture was washed out prior to addition of MTT reagent. Then, the cell was incubated with MTT (0.5 mg/mL) for 4 h. DMSO was then added to dissolve the precipitate after washing out the MTT, and O.D. values were read at 570 nm in a microplate reader.

2.6. Proliferation Assay. Splenic lymphocytes (4 × 10⁵ cells/well) and CT-F or five procyanidin oligomers compounds in 96-well plates were cultured in triplicate for 48 h by using ConA (2 μg/mL) or LPS (10 μg/mL). Cells were pulsed at 0.25 μCi/well of [3H]-thymidine for 8 h before the end of the culture and then harvested onto glass fiber filters. Incorporated radioactivity was then measured by using a beta scintillation counter (MicroBeta Trilux, PerkinElmer Life Sciences, Boston, MA, USA).

2.7. Cytokine Measurement and DNFB-Induced DTH Reactions of CTD-1. Since the results of compounds activity screening showed that CTD-1 had the best immunosuppression effect on ConA-induced splenocyte proliferation, only CTD-1 was used for the cytokine and DNFB assay out of 5 compounds. Generally, splenocyte cells (4 × 10⁵ cells/well) were incubated with 2 μg/mL ConA and CTD-1 in 24-well plates. After 48 h, culture supernatants were harvested and stored at −20°C. IL-2, IFN-γ concentrations in supernatant were assessed by performing ELISA according to the manufacturer’s instructions.
DNFB-induced DTH reaction of CTD-1 was examined according to a previously described method [15]. BALB/c mice were initially sensitized with 0.5% DNFB dissolved in acetone–olive oil (4:1) on each hind foot on days 0 and 1. On day 9, mice were challenged with 0.4% DNFB on both sides of their left ear. Vehicle, CTD-1 (3.3, 10, and 20 mg/kg), was administered to each group p.o. after the first day of initial sensitization. Five female BALB/c mice were prepared for each group. Ear swelling indicated the difference between the weights or thickness of the left and right ear patches, which were obtained from 8 mm punches 24 h after challenge.

2.8. Statistical Analysis. Results were expressed as mean ± SD, one-way analysis of variance (ANOVA) followed by Dunnett’s test analysis was performed, and a value of $P < 0.05$ was considered significant.

3. Results

3.1. Structural Elucidation of Procyanidin Oligomer Compounds 1 to 5 (Figure 1). Separation of CT-F by ODS and Toyopearl HW-40 columns led to the isolation of three A-type procyanidin oligomers (compounds 1–3), whereas separation of CC-F using the same columns led to the isolation of two B-type procyanidin oligomers (compounds 4 and 5). The structure of procyanidin oligomer compounds 1 to 5 was shown in Figure 1. The purity of each compound was detected more than 95% by using HPLC method (Supplementary Material available online at http://dx.doi.org/10.1155/2014/365258).

Compound 1 was obtained as an off-white freeze-dried powder: [$\alpha$]$^2$D + 65° ($c = 0.1$, MeOH). The ESI-MS spectra of 1 showed the molecular ion [M-H]$^-$ at $m/z$ 863, which indicates an A-type interflavanoid linkage procyanidin trimer. $^{13}$C NMR (CD$_3$OD, 100 MHz) spectrum of 1 exhibited ketal resonance at $\delta_C$ 100.0, and the $^1$H NMR (CD$_3$OD, 400 MHz) spectrum yielded AB system protons at $\delta_H 3.31$ (IH, $d$, 3.4 Hz) and 4.17 (IH, $d$, 3.4 Hz), which indicates the presence of an A-type double-linked structure [16]. Carbon signals at $\delta_C$ 78.9 and 80.3 were consistent with C-2 resonances of epicatechin moieties. $^1$H and $^{13}$C NMR spectroscopic data (Supplementary Material) were compared with those in previous studies and revealed that 1 was epicatechin-(4$\beta$→8, 2-O-7)-epicatechin-(4$\beta$→8)-epicatechin (cinnamtannin B1, CTB-1) [17, 18].

Compound 2 was obtained as an off-white freeze-dried powder: [$\alpha$]$^2$D + 108° ($c = 0.1$, MeOH). The ESI-MS spectra of 2 also showed the molecular ion [M-H]$^-$ at $m/z$ 863, which indicates an A-type procyanidin trimer. The NMR data of 2 were similar to those of 1, except that aliphatic proton resonances appeared at $\delta_C 83.3, 70.1$, and $30.7$ and $\delta_H 3.94$ (IH, $d$, 9.1 Hz), 3.67 (m), 3.04 (IH, $dd$, 16.2 Hz, 6.2 Hz), and 2.41 (IH, $dd$, 16.2 Hz, 10.1 Hz), which are consistent with the terminal unit as a catechin moiety. $^1$H and $^{13}$C NMR spectroscopic data (Supplementary Material) were compared with those in previous studies and revealed that 2 was epicatechin-(4$\beta$→8, 2-O-7)-epicatechin-(4$\beta$→8)-catechin (cinnamtannin D1, CTD-1) [19, 20].

Compound 3 was obtained as an off-white freeze-dried powder: [$\alpha$]$^2$D + 46° ($c = 0.1$, MeOH). The ESI-MS spectra of 3 exhibited a molecular ion [M-H]$^-$ at $m/z$ 1151, which suggests an A-type procyanidin tetramer. The $^1$H NMR (CD$_3$OD, 400 MHz) spectrum of 3 showed the presence of two sets of meta-coupled protons at $\delta_H 6.00$ (IH, $d$, 2.0 Hz) and 6.08 (IH, $d$, 2.0 Hz) and $\delta_H 5.95$ (IH, $d$, 2.0 Hz) and 5.90 (IH, $d$, 2.0 Hz) along with a single proton at $\delta_H 6.10$ (IH, $s$), which indicates C-4$\alpha$-C-8$'$ (C-ring, D-ring) and C-4$''$-C-6'(I-ring, D-ring) linkages. In the $^{13}$C-NMR spectrum, the presence of three carbon signals at $\delta_C 78.7$, 80.2, and 76.6 due to the C-2 of each flavan-3-ol unit suggests that the other flavan-3-ol units were epicatechin moieties. Compound 3 was identified as epicatechin-(4$\beta$→8, 2-O-7)-epicatechin-(4$\beta$→6)-epicatechin-(4$\beta$→8)-epicatechin (parameritannin A1, PA-1) based on a direct comparison between its spectral data (Supplementary Material) and those of previous studies [21].

Compound 4 was obtained as light brown freeze-dried powders: [$\alpha$]$^2$D + 26° ($c = 0.1$, MeOH). ESI-MS spectra showed the molecular ion [M-H]$^-$ at $m/z$ 577, which suggested that 4 was a B-type procyanidin dimer. Carbon signals at $\delta_C$ 77.1, 79.7 (CD$_3$OD, 100 MHz) were consistent with the C-2 resonances of epicatechin moieties [22]. The spectral data of 4 (Supplementary Material) were consistent with epicatechin-(4$\beta$→8)-epicatechin (procyanidin B2, PB-2), which was also recorded in literature [23, 24].

Compound 5 was obtained as a light brown freeze-dried powder: [$\alpha$]$^2$D + 82° ($c = 0.1$, MeOH). ESI-MS spectra showed a single molecular ion [M-H]$^-$ at $m/z$ 865, which indicates that it was a B-type procyanidin trimer. Carbon signals at $\delta_C$ 77.1, 77.1, and 79.7 were consistent with the C-2 resonances of epicatechin moieties. $^1$H and $^{13}$C NMR spectroscopic data (Supplementary Material) were compared with those in previous studies and revealed that 5 was epicatechin-(4$\beta$→8)-epicatechin-(4$\beta$→8)-epicatechin (procyanidin C1, PC-1) [24, 25].

3.2. Characterization of TAPOs from CT-F. As shown in Figure 2, the retention time values of the main chromatographic peaks in CT-F were at 6.7, 9.2, 11.2, 12.7, 13.4, 15.6, and 17.4 min. These peaks were marked as 1 to 7. In comparison to the reference compounds isolated from two cinnamon barks, the peaks were identified as follows: 1 was PA-1; 2 was PB-2; 3 was CTD-1; 4 was CTB-1; 5 was PC-1. Peaks 6 and 7 were identified as A-type procyanidin trimers according to ESI-MS data that showed the presence of molecular ions at $m/z$ 863 in the negative ion mode (Supplementary Material). Hence, CT-F may primarily contain A-type procyanidin because the main peaks in CT-F were CTD-1 and CTB-1, which are A-type procyanidin trimers.

3.3. CT-F Cytotoxicity on Splenocytes and Inhibition of ConA-Induced Splenocyte Proliferation. The immunosuppressive effects of CT-F were evaluated in vitro by ConA-induced splenocyte proliferation. The results showed that CT-F has no cytotoxic effect on splenocytes at concentrations of 3.1 $\mu$g/mL to 50 $\mu$g/mL (Figure 3(a)). Figure 3(b) revealed that CT-F had significant inhibition on splenocyte proliferation under ConA induction in a concentration-dependent manner. The
3.4. Evaluating Immunosuppressive Effects of Compounds 1 to 5. The immunosuppressive effects of the five procyanidins were determined using the same method (Figure 4). It was found that all five test compounds have no cytotoxic effect at concentrations from 0.16 µM to 20 µM (Figure 4(a)). Figure 4(b) showed that, even at a low concentration of 0.16 µM, CTD-1 can inhibit ConA-induced splenocyte proliferation by 40%, suggesting that CTD-1 produced the best effects of all compounds. Other A-type procyanidin oligomers CTB-1 and PA-1 significantly suppressed ConA-induced splenocyte proliferation at 20 µM. However, B-type procyanidin dimer PB-2 had no inhibitory effect even at 20 µM. Meanwhile, B-type procyanidin trimer PC-1 had weakly effect on ConA-induced splenocyte proliferation, only reducing it by 37% at a high concentration of 20 µM.

3.5. CTD-1 Inhibition of Mitogen-Induced Splenocyte Proliferation. In order to further evaluate the immunosuppressive effect of CTD-1, two in vitro models, ConA and LPS induced splenocyte proliferation, were used in this experiment. The proliferation was suppressed in the CTD-1 treated splenocyte under ConA or LPS induction in a concentration-dependent manner (Figures 5(b) and 5(c)). The values of 4.38 ± 0.12 µM
and 6.62 ± 1.04 μM correspond to two models induced by ConA and LPS. So the different of two values is reasonable.

3.6. Effects on Cytokine Production and DNFB-Induced DTH Response of CTD-1. The effects of CTD-1 on the production of Th1-type cytokines IL-2 and IFN-γ and effect on DTH responses in BALB/c mice were examined. As shown in Figure 6, IL-2 and IFN-γ productions were significantly decreased when the splenocyte cultures were exposed to CTD-1. In in vivo experiment, in DNFB-induced DTH, CTD-1 significantly suppressed ear weight (thickness) by 8.9% (7.6%), 29.4% (10.7%), and 36.2% (29.2%) at the dosage of
3.3, 10, and 20 μM, respectively. These results suggested that CTD-1 might protect hosts from inflammatory reactions via strong inhibition of T cell functions (Figure 7).

4. Discussion

Since procyanidin oligomers compounds are difficult to purify, the regulating immune functions of cinnamon were mainly focused on extracts but not purified compounds in the previous studies [7, 8]. In this paper, the main procyanidin oligomers in cinnamon were successfully purified with the Toyopearl HW-40 and ODS-A-HG C18 gel chromatographic column. The HW-40 gel shows good ability in separation of the procyanidin oligomers with different degree of polymerization, whereas ODS CI8 gel is good at separating the oligomers with different polarity and is also available for the isolation of isomers such as CTB-1 and CTD-1. The combined application of these chromatographic supports provides a good method for the isolation and purification of procyanidin oligomers, resulting in sufficient quantities of purified single procyanidin oligomers for active assay.

Our findings indicate that A-type procyanidin oligomers have better immunosuppressive effects than B-type procyanidin oligomers. Similar A-type procyanidin trimers, CTD-1 and CTB-1, showed significantly different effects. The linkage pattern between procyanidin units and the spatial...
configuration of hydroxyl group on C-ring of terminal unit might play an important role in immunosuppression.

Moreover, CTD-1 exhibits significant immunosuppressive effects in vitro and in vivo, which include suppression of lymphocyte proliferations induced by ConA and LPS, inhibition of Th1-type cytokines IL-2 and IFN-γ production, and inhibition of DTH response. These would result in the decrement of cellular and humoral immune responses. The immunosuppression appears to be mediated by T cells, indicating that the immune regulation effect of CTD-1 might inhibit T cell functions. Furthermore, CTD-1 possessed relative high immunosuppressive activities with low cytotoxicity.

Cytokines play an important role in the immune system and in immunomodulatory potential targets [26, 27]. IFN-γ plays a pivotal role in immune inflammatory reactions. IFN-γ can activate macrophages, which subsequently synthesizes TNF-α and proinflammatory chemokines to maintain inflammation [14]. CTD-1 inhibited IFN-γ, IL-2 productions, which suggested its possible therapeutic role in treating diseases that are associated with synthesis/release of these cytokines. To assess the immunosuppressive effects of CTD-1 on cellular and humoral immunity, a DTH model was used in vivo. The inhibitory effect of CTD-1 on T cell functions was confirmed in a murine DTH model. Ear swelling is
recognized as the primary result of functions of antigen-specific in vivo CD4+ T cell response in DTH [28, 29]. Administration of CTD-1 strongly inhibited DNFB-induced ear swelling, which indicates its suppressive effect on T-celldependent immune responses. Thus, CTD-1 may protect hosts from inflammatory reactions via strong inhibition of T cell functions.

5. Conclusions

CT-F and CTD-1 exhibit low toxicity and have significant immunosuppressive effects, which can be attributed to their inhibition of T cell functions. In the meantime, our results indicate that the treatment of RA using cinnamon may be due in part to the procyanidin oligomers especially A-type. CTD-1 is a good candidate as a potential therapeutic agent for inflammatory and autoimmune diseases.

Abbreviations

ConA: Concanavalin A  
LPS: Lipopolysaccharide  
DNFB: 2,4-Dinitrofluorobenzene  
DTH: Delayed-type hypersensitivity  
RA: Rheumatoid arthritis  
TCM: Traditional Chinese medicine  
TAPOs: A-type procyanidin oligomers  
PB-2: Procyanidin B2  
CTB-1: Cinnamtannin B1  
CTD-1: Cinnamtannin D1  
PA-1: Parameritannin A1  
PC-1: Procyanidin C1.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

This research was supported by the National Natural Science Foundation of China (no. 81173518), the Program for Professors of Special Appointment (Eastern Scholar) in Shanghai Institutions of Higher Learning (2012–90), the Shanghai Municipal Education Commission (A1-20140354), the "Xinlin" scholars, and the outstanding team training plan of Shanghai Municipal Education Commission (A1-20140354), Shanghai Institutions of Higher Learning (2012–90), the Professors of Special Appointment (Eastern Scholar) in Shanghai Municipal Education Commission (A1-20140354), the Shanghai Municipal Education Commission (A1-20140354), the "Xinlin" scholars, and the outstanding team training plan of SHUTCM.

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