Inhibitory Effects of the Flavonoids Isolated from Waltheria indica on the Production of NO, TNF-α and IL-12 in Activated Macrophages

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Received December 9, 2004; accepted January 11, 2005

Three flavonoids were isolated from the whole plants of Waltheria indica and biological properties investigated. On the basis of their spectroscopic data, these compounds were identified as (−)-epicatechin, quercetin, and tiliroside. These flavonoids significantly and dose-dependently inhibited the production of the inflammatory mediator nitric oxide (NO), and the cytokines (tumor necrosis factor (TNF)-α and interleukin (IL)-12), in lipopolysaccharide (LPS) and interferon (IFN)-γ activated murine peritoneal macrophages, without displaying cytotoxicity. The order of inhibitory activity was quercetin > tiliroside > (−)-epicatechin. Furthermore, peritoneal macrophages were pre-activated with LPS/IFN-γ for 24 h, and the inhibitory effects of the above mentioned isolates on the production of NO were determined after a further 24 h, to address the possible mechanisms of their action. The present study supports the use of W. indica for the treatment of inflammatory diseases in traditional medicine.

Key words Waltheria indica; flavonoid; nitric oxide; tumor necrosis factor-α; interleukin-12

Inflammation is normally a localized, protective response to tissue injury. Macrophages play major roles in the immunity and inflammatory responses involved in host defense. In macrophages, bacterial lipopolysaccharide (LPS) or in combination with interferon (IFN)-γ, are the best stimuli characterized to induce the inflammatory mediators, cytokines, oxygen and nitrogen species. Nitric oxide (NO) is a short-lived free radical and intracellular messenger which is produced by the oxidation of L-arginine catalyzed by NO synthase (NOS) that mediates a variety of biological functions. In the NOS family, inducible NOS (iNOS) is particularly involved in pathological overproduction of NO, which is known to play a central role in inflammatory and immune reactions. However, excessive production of NO may cause tissue damage. In inflammatory diseases such as rheumatoid arthritis, excessive NO production by activated macrophages has been observed. On the other hand, cytokines are soluble hormone-like protein mediators. The tumor necrosis factor (TNF)-α and interleukin (IL)-12 are the major macrophage-derived inflammatory cytokines. Although these cytokines play important roles in the normal physiology of cells and organs, they are distinguished for their activities associated with immune response, inflammation, tissue injury or repair, and organ dysfunction. Thus, agents that block the excessive productions of TNF-α, IL-12 and/or NO might be beneficial in the treatment of inflammatory diseases.

Many natural compounds isolated from different parts of plants have shown anti-inflammatory and immunomodulatory activities. Waltheria indica L. (Stereulaceae) is a short-lived shrub in India, Taiwan and Southeast Asia that normally is drunk as a tea or juice. As an alternative medicine, it has been used in the treatment of diverse conditions of inflammation, rheumatism, circulatory problems, and immune system deficiencies. However, there is no data in the literature about W. indica phytochemicals or their pharmacological activities. To validate the use of W. indica as an anti-inflammatory drug in folk medicine, this study was undertaken to investigate its constituents and their effects on the production of NO and cytokines (TNF-α and IL-12) in murine peritoneal macrophages activated by LPS and IFN-γ. The effects of W. indica isolates on LPS/IFN-γ pre-activated NO synthesis were also studied to address the possible mechanisms of their action.

MATERIALS AND METHODS

Materials Whole plants of W. indica L. were collected in December 2002 from Tirumala Hills, A. P. S. India. A voucher specimen (YKR-023) has been deposited in the Herbarium of the Department of Botany, Sri Venkateswara University, Tirupati. The samples were shade dried and milled to powder form, and were then kept in an air-tight brown bottle until use. LPS (from Escherichia coli 055:B5), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), IFN-γ (10 U/ml), and Griess reagent were purchased from Sigma Chemical (St. Louis, MO, U.S.A.). RPMI-1640 medium, Hank’s balanced salt solution (HBSS), penicillin, streptomycin, and fetal calf serum were purchased from Gibco BRL (Grand Island, NY, U.S.A.).

Extraction and Isolation The air-dried whole plants of W. indica (1.2 kg) were pulverized using a milling machine and extracted with 80% aq. EtOH (4 l×5) under reflux. The combined extract was filtered, and the filtrate was concentrated under reduced pressure to give about 48 g of dark brown syrup (4.0% based on the dry weight). The crude extract was suspended in distilled H₂O, defatted with n-hexane, and then apportioned with chloroform and n-butanol sequentially to yield the respective solvent extracts. The chloroform extract (22 g, 1.83% w/w) was chromatographed on a silica gel (5×90 cm, 0.063—0.200 mesh) column by eluting with a gradient of n-hexane/EtOAc (8:2 to 100% EtOAc). Twenty five column fractions were collected and analyzed by TLC (n-hexane–EtOAc). Fractions with similar TLC patterns were combined, and rechromatographed on a silica gel column to yield (−)-epicatechin [900 mg, 0.075% (w/w)], and quercetin [60 mg, 0.05% (w/w)]. The concentrated n-butanol extract (14 g, 1.17% w/w) was subjected to repeated column chromatography on silica gel and eluted...
with a gradient of CHCl₃ and MeOH (7:3) to give a pure compound, kaempferol-3-O-(6′-p-coumaryl)-β-d-glucoside, tiliroside [39 mg, 0.0033% (w/w)].

**Structure Elucidation** The isolates were characterized by the following methods: UV spectra were recorded on a Varian Cary Win UV-50 spectrophotometer. ¹H-, ¹³C-NMR spectra were recorded on a Bruker Avance 500 spectrometer operating at 500.15 MHz, 125.20 MHz, respectively in DMSO-d₆ and CDCl₃. ESI-MS were recorded on a Thermo-Finnigan LCQ Advantage system. Column chromatography (CC) separations were carried out using silica gel 60 (0.063—0.200 mm) supplied by E. Merck. The structures of (−)-epicatechin, quercetin, and tiliroside (Fig. 1), were elucidated by detailed ¹H- and ¹³C-NMR spectroscopy and by comparison of the spectral data with those published values,⁸⁻¹⁰ and their molecular masses were 290, 302, and 594 g/mol, respectively.

**Animals** Female Balb/c mice were obtained from the Animal Center of the College of Medicine, National Taiwan University and maintained under constant conditions (temperature 20±2°C, humidity: 40—60%, 12 h light/dark cycle), in the Animal Center of China Medical University. This study was conducted in accordance with the standards outlined in the Guidelines for the Care and Use of Laboratory Animals of China Medical University, Taichung, Taiwan.

**Macrophage Cultures** Mouse peritoneal excluded macrophages were obtained from mice by lavage with 10 ml of cold HBSS per mouse at 3 d after i.p. injection of 2 ml 3% thioglycollate in saline (1.5 ml per mouse). Cells were seeded in 96-well cluster plates at a density of 2 x 10⁶ cells/ml and incubated at 37 °C in humidified 5% CO₂/95% air to allow macrophage adherence. Two hours later, the non-adherent cells were removed by washing with warmed PBS and the remaining cells (90% macrophages, judged by non-specific esterase stain) were used for further experiments. Test compounds were dissolved in DMSO, while the control cells were treated only with solvent and the final concentration never exceeded 0.1% (v/v). Cell-free supernatants were harvested after 24 h incubation with the stimuli and assayed for nitrite and cytokines.

**MTT Assay for Cell Viability** Cell viability studies were performed in 96-well plates. MTT in PBS (0.1 mg) was added to each well and then incubated at 37 °C for 4 h. The MTT formazan (1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazon) crystals were dissolved using acidified iso-propanol (0.1 N HCl) and mixed at room temperature. After 20 min, the index of cell viability was calculated by measuring the optical density (OD) with a microplate reader (BIORAD, model 3550, U.S.A.) at 540 nm (OD₅₇₀—620). The mean OD value of the content of four wells was used for assessing the cell viability expressed as % of control.

**Nitrite Determination** Nitrite accumulation in culture medium was measured as an indicator of NO production based on the Griess reaction. Briefly, 100 μl of cell culture medium was mixed with 100 μl of Griess reagent [equal volumes of 1% (w/v) sulphanilamide in 5% (v/v) phosphoric acid and 0.1% (w/v) naphthylenediamine-HCl], incubated at room temperature for 10 min, and then the absorbance was measured at 540 nm in a spectrophotometer. Cells were incubated with medium containing LPS (2 μg/ml) plus IFN-γ (10 U/ml) in the presence or absence of various concentrations of test compounds for 24 h. The amount of nitrite in the samples was determined versus a sodium nitrite standard curve and nitrite production was measured. The results are the mean of three independent experiments.

**Measurement of TNF-α and IL-12 Production** TNF-α and IL-12 levels in macrophage culture medium were determined by ELISA kits that are specific against murine cytokines according to the manufacturer’s instruction with a limit of detection of 15.6 pg/ml.

**Statistical Analysis** Statistical analysis was performed by analysis of variance (ANOVA) followed by Dunnett’s multiple comparison test. Data are shown as means±S.D. The significant difference was set at *p<0.05; **p<0.01.

**RESULTS**

The sequential fractionation of *W. indica* crude EtOH extract with chloroform and n-butanol yielded the respective solvent extracts. The chloroform fraction was chromatographed on a silica gel column using the n-hexane/ EtOAc gradient solvent system to obtain (−)-epicatechin and quercetin. The n-butanol fraction was chromatographed by conventional isolation procedures to afford tiliroside (Fig. 1). These three compounds were isolated for the first time from this plant.

To determine the effects of *W. indica* isolates on NO production in murine macrophages, the cells were treated with LPS (2 μg/ml) and IFN-γ (10 U/ml) in the presence or absence of various concentrations of test compounds for 24 h. As shown in Fig. 2, (−)-epicatechin, quercetin, and tiliroside showed an inhibitory effect on LPS/IFN-γ-induced NO production in a dose dependent manner based on three separate experiments (Fig. 2, open column). Quercetin was significantly the most active, followed by tiliroside and (−)-epicatechin in this study. This effect was more evident at 12.5 μM of quercetin, 50 μM of tiliroside, and 200 μM of (−)-epicatechin reduced to 90%, 63%, and 34%, respectively, of the NO production. Under the experimental conditions described above, no cytotoxic effects were observed during the concentration range studied by MTT assay.
stimulated by LPS/IFN-γ of the test compounds to the peritoneal macrophage cultures production of TNF-α level of concentration.

NO by 22 and 25%, respectively, at their maximum tested failed to inhibit, while (−)-epicatechin and tiliroside reduced the NO production in macrophages that had been pre-activated. Interestingly, quercetin added 24 h after the addition of LPS/IFN-γ did not significantly inhibit the NO production from macrophage cells, in which iNOS was already fully induced. Therefore, it is suggested that inhibition of NO production by quercetin may be due to its reduction of iNOS enzyme expression, at least in part. It is well known that the

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (µM)</th>
<th>Nitric oxide synthesis (%)</th>
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<tbody>
<tr>
<td>Epicatechin</td>
<td>25</td>
<td>105.0±2.2</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>106.4±0.5</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>100.6±1.9</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>78.1±3.7</td>
</tr>
<tr>
<td>Quercetin</td>
<td>2.5</td>
<td>104.2±3.1</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>101.3±1.9</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>99.7±2.2</td>
</tr>
<tr>
<td></td>
<td>12.5</td>
<td>98.5±2.6</td>
</tr>
<tr>
<td>Tiliroside</td>
<td>20</td>
<td>104.0±2.2</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>89.1±1.9</td>
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<tr>
<td></td>
<td>40</td>
<td>78.2±2.6</td>
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<tr>
<td></td>
<td>50</td>
<td>75.6±3.1</td>
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The assay procedure was described in the experimental methods. The data represent the mean±S.D. of triplicate cultures.

with an inhibition percentage at 12.5 µM of quercetin of 88% and 87%; with 50 µM of tiliroside of 68% and 64%; and with 200 µM of (−)-epicatechin of 28% and 25%, respectively.

DISCUSSION

The plant *W. indica* has been used traditionally as a medicine to treat a broad range of diseases, including anti-inflammatory and immune system deficiencies. However, to our knowledge the anti-inflammatory activity of the components of this plant are not well defined. In our continuing search for novel anti-inflammatory agents from natural sources, and in order to validate the use of *W. indica* as an anti-inflammatory drug in traditional medicine, we isolated its constituents and evaluated their effects on the production of NO and cytokines (TNF-α and IL-12) in LPS/IFN-γ-activated mouse macrophages. We found that the flavonoids, (−)-epicatechin, quercetin, and tiliroside isolated from *W. indica* extracts are able to inhibit the production of three major macrophage-derived inflammatory mediators in a dose-dependent manner (Fig. 2).

Flavonoids have been identified as either simple or complex glycosides in plants, and a human has been estimated to consume approximately 1 g of flavonoids/day. These polyphenolic compounds and their sugar derivatives display a remarkable spectrum of biological activities including anti-inflammation. Furthermore, a flavonoid derivative flavopiridol has been found to inhibit cyclin-dependent kinases, induce apoptosis, suppress inflammation, and modulate the immune response. Based on the inhibitory values the order of potency for the inhibition of NO production by peritoneal macrophages was quercetin > tiliroside > (−)epicatechin, without any evidence of cytotoxic effect. The inhibitory activity of these compounds resulted from the inhibition of iNOS expression and/or activity. Accordingly, quercetin did not change, and (−)-epicatechin and tiliroside reduced the NO production in macrophages that had been pre-activated. Interestingly, quercetin added 24 h after the addition of LPS/IFN-γ did not significantly inhibit the NO production from macrophage cells, in which iNOS was already fully induced. Therefore, it is suggested that inhibition of NO production by quercetin may be due to its reduction of iNOS enzyme expression, at least in part. It is well known that the
widespread flavonol quercetin has long been known as a protein-tyrosine kinase inhibitor, to modulate the pro-survival Akt/PKB and ERK1/2 signaling cascade, and to inhibit the cell signaling kinase Cdk2.\textsuperscript{13,14} Furthermore, in a more recent study quercetin has been reported to be an inhibitor of IL-12 signaling through the JAK-STAT pathway in T lymphocyte.\textsuperscript{15} Our results also support the earlier findings that quercetin significantly inhibited the production of NO\textsubscript{2} in LPS-stimulated macrophages.\textsuperscript{4,16—18}

Cytokines are central mediators of pathological processes and they are involved in necrosis, inflammation, apoptosis and fibrosis.\textsuperscript{19} Elevated levels of cytokines such as IL-12 and TNF-\alpha, are linked to many disorders including Crohn’s disease and psoriasis. In addition, these proinflammatory cytokines play an essential role in the pathogenesis of rheumatoid arthritis and osteoarthritis.\textsuperscript{20} The inhibition of cytokines, in particular TNF-\alpha, has been successful in several clinical trials for the treatment of rheumatoid arthritis.\textsuperscript{21} Further, IL-12 itself is a potent inducer of IFN-\gamma production, another cytokine related to lethality in endotox shock. The importance of down-regulating IL-12 is reflected in the number of diseases characterized by excessive IL-12 production.\textsuperscript{22} Although some previous reports suggest that tiliroside might modulate hepatoprotective, anti-phospholipase, and anti-complement activities,\textsuperscript{23—25} there has been no study so far showing the inhibitory activities of IL-12. The results of this study showed that the isolates of \textit{W. indica} significantly inhibited the LPS/IFN-\gamma-induced macrophage derived TNF-\alpha and IL-12.

In conclusion, the present investigation on the whole plants of \textit{W. indica} resulted in the isolation of three flavonoids for the first time. These compounds significantly and dose-dependently inhibited the production of the inflammatory mediators NO, TNF-\alpha and IL-12 in activated macrophages. The results of the present study provide scientific evidence supporting the use of \textit{W. indica} as an anti-inflammatory remedy in folk medicines.

Acknowledgements This research was supported by the National Science Council of Taiwan (NSC 93-2811-M-324-001). In addition, a part of this investigation was supported by a research grant from China Medical University (CMU-93-M-06).

REFERENCES