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Anti-inflammatory effects of several plant extracts on porcine alveolar macrophages in vitro

Y. Liu,* M. Song,* T. M. Che,* D. Bravo,† and J. E. Pettigrew*2

*Department of Animal Sciences, University of Illinois at Urbana-Champaign, Urbana; and †Pancosma SA, Geneva, Switzerland

ABSTRACT: Certain plant extracts are bioactive substances of some foods or traditional herbs, known to possess antioxidant, antibacterial, and perhaps immunoregulatory effects. This study investigated the in vitro anti-inflammatory effects of 7 plant extracts (anethol, capsicum oleoresin, carvacrol, cinnamaldehyde, eugenol, garlicon, and turmeric oleoresin) on porcine alveolar macrophages collected from weaned pigs (n = 6 donor pigs) by bronchoalveolar lavage. The experimental design for this assay was a 2 (with or without 1 μg lipopolysaccharide (LPS)/mL) × 5 (5 different amounts of each plant extract) factorial arrangements in a randomized complete block design. The application of plant extracts were 0, 25, 50, 100, and 200 μg/mL, except for cinnamaldehyde and turmeric oleoresin, which were 0, 2.5, 5, 10, and 20 μg/mL. The 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) assay was used to determine the number of live cells, Griess assay was applied to detect nitric oxide (NO) production, and ELISA was used to measure tumor necrosis factor-α (TNF-α), IL-1β, transforming growth factor-β (TGF-β), and IL-10 in the cell culture supernatants of macrophages. The LPS increased (P < 0.001) the secretion of TNF-α, IL-1β, and TGF-β. Without LPS, anethol and capsicum oleoresin increased (linear, P < 0.001) cell viability of macrophages, whereas other plant extracts reduced (linear, P < 0.001) it. Anethol, capsicum oleoresin, and carvacrol enhanced (linear, P < 0.001) the cell proliferation of LPS-treated macrophages. Without LPS, anethol, capsicum oleoresin, cinnamaldehyde, or turmeric oleoresin stimulated TNF-α secretion, whereas all plant extracts except eugenol enhanced IL-1β concentration in the supernatants of macrophages. However, all plant extracts suppressed (linear, P < 0.001) TNF-α, and all plant extracts except turmeric oleoresin decreased (linear, P < 0.05) IL-1β secretion from LPS-treated macrophages. Anethol and capsicum oleoresin decreased (linear, P < 0.001) TGF-β from macrophages in the absence of LPS, but the other plant extracts increased it. Anethol, capsicum oleoresin, and carvacrol also suppressed (linear, P < 0.001) TGF-β from macrophages with LPS stimulation; the other plant extracts enhanced or did not affect it. The anti-inflammatory cytokine, IL-10, was not detected in any supernatants. Only very low amounts of NO were detected in the supernatants of macrophages. In conclusion, the TNF-α results indicate all plant extracts tested here may have anti-inflammatory effects to varying degrees.

Key words: alveolar macrophage, cell viability, cytokines, plant extracts, weaned pigs

INTRODUCTION

Certain plant extracts from traditional herbs are considered to have antioxidant, antibacterial, and per-
haps immunoregulatory effects (Lee et al., 2004). The active components of plant extracts also can be synthesized in pure form. Many plant extracts have been shown to promote growth in pigs, maybe partly due to the ability of plant extracts to modulate the immunity of pigs (Sads and Bilkei, 2003; Janz et al., 2007). These specific effects need to be clarified. In particular, it is useful to know the immune-modulating impact of specific plant extracts in vitro first. Macrophages are involved in the innate immune response through phago-

cytosis or production of a variety of compounds, like cytokines or nitric oxide (NO; Dempsey et al., 2003). Tumor necrosis factor-α (TNF-α) and IL-1β are proinflammatory molecules whose secretion can be potently induced by lipopolysaccharide (LPS; Alexander and Rietschel, 2001). Overproduction of these cytokines and NO might cause inflammatory diseases (Bogdan, 2001). Macrophages also release anti-inflammatory cytokines, such as IL-10 (Opal and DePalo, 2000). Eugenol and allicin had potential anti-inflammatory effects shown as inhibition of TNF-α and IL-1β secretion from LPS-induced human or rat cells (Lang et al., 2004; Lee et al., 2007). Previous studies from Lee et al. (2005) and Li et al. (2006) reported that cinnamaldehyde and eugenol can suppress NO release and inducible nitric oxide synthase expression in LPS-treated murine macrophages. Most of these in vitro experiments have been conducted in human, mouse, or rat cells. However, the potential anti-inflammatory effects of plant extracts on porcine cells remain to be elucidated.

The objective of this study was to investigate the effects of 7 plant extracts on the inflammatory response in porcine alveolar macrophages. The results may indicate whether there is a potential for these plant extracts to be evaluated further for possible application as dietary additives.

MATERIALS AND METHODS

The protocol for this experiment was reviewed and approved by the Institutional Animal Care and Use Committee at the University of Illinois at Urbana-Champaign.

Materials

Seven plant extracts (anethol, capsicum oleoresin, carvacrol, cinnamaldehyde, eugenol, garlic, and turmeric oleoresin) were provided by Pancosma, SA (Geneva, Switzerland). Some of the products tested were purified extracts, whereas others were chemically synthesized: generally, plant extracts contain more compounds than pure synthesized compounds. The broad terminology plant extracts is used here to represent all compounds and more than 95% pure. Capsicum and turmeric are extracted oleoresins, standardized to 6% capsaicin and dihydrocapsaicin and 98% curcinomides, respectively. Garlicon is a botanical extract from garlic, standardized to 40% propyl thiosulfonates. Before conducting the experiment, all plant extracts were first dissolved in dimethyl sulfoxide (DMSO) and were further diluted with the sterile culture medium RPMI-1640 (Roswell Park Memorial Institute medium, HyClone Laboratories, Inc., Logan, UT) containing 10% heat-inactivated fetal bovine serum (FBS; HyClone Laboratories, Inc., Logan, UT) and antibiotics, including 100 IU penicillin/mL and 100 μg streptomycin/mL (Mediatech, Inc., Manassas, VA). The final concentration of DMSO in the medium did not exceed 0.05%. Lipopolysaccharide (from Escherichia coli 0111:B4) was purchased from Sigma Co. (St. Louis, MO). Vybrant MTT Cell Proliferation Assay Kits were purchased from Molecular Probes Inc. (Eugene, OR). The Griess Reagent System was purchased from Promega Corp. (Madison, WI). Porcine TNF-α, IL-1β, transforming growth factor-β (TGF-β), and IL-10 ELISA kits were purchased from R&D Systems, Inc. (Minneapolis, MN).

Collection of Porcine Alveolar Macrophages

Eighteen clinically healthy donor pigs were used as donors of porcine alveolar macrophages. All pigs were healthy and around 6 wk old and 10 kg BW. The BW of pigs was not considered as an effector in the in vitro study. Each group of 6 pigs was used to test 2 or 3 plant extracts. Pigs were anesthetized by intramuscular injection of a 1-mL combination of telazol, ketamine, and xylazine (2:1:1) per 23.3 kg BW. The final mixture contained 100 mg telazol, 50 mg ketamine, and 50 mg xylazine in 1 mL (Fort Dodge Animal Health, Fort Dodge, IA). After anesthesia, pigs were euthanized by intracardiac injection with 78 mg sodium pentobarbital (Sleepaway; Henry Schein, Inc., Indianapolis, IN) per 1 kg of BW.

Porcine alveolar macrophages from lungs were obtained by bronchoalveolar lavage by the following procedures (Baarsch et al., 1991): briefly, lungs with intact trachea were removed immediately after euthanizing pigs and 150 mL PBS was poured into them through the trachea. After massaging the lungs for about 30 to 60 s, the lavage fluid was filtered through a double layer of sterile gauze into 50-mL conical centrifuge tubes and then pelleted by centrifuging at 400 × g for 15 min at room temperature. The pelleted cells were washed twice with Hank’s balanced salt solution (pH of 6.8; Hyclone Laboratories, Inc., Logan, UT) and were resuspended in 5 mL of the culture medium RPMI-1640 with FBS and antibiotics (pH of 7.0). Live cells were stained with trypan blue dye exclusion (Sigma-Aldrich Co., St. Louis, MO) and were counted using a hemocytometer (Fisher Scientific, Inc., Pittsburgh, PA). The final cell concentration was adjusted to 1 × 10^5 cells/mL. The viability of the cells was greater than 97%. In this paper, we use the term “porcine alveolar macrophages” because the majority (93% to 97%) of bronchoalveolar lavage fluid cells are macrophages (Dickie et al., 2009).
**Cell Culture and Experimental Design**

Porcine alveolar macrophages were cultured in 48- or 96-well plastic tissue culture plates at a density of 6 × 10^4 cells/well in a 48-well plate and 1 × 10^4 cells/well in a 96-well plate. All plates were incubated overnight at 37°C in a humidified 5% CO2 incubator to allow macrophages to attach to the bottom. The nonadherent cells were washed away with warm Hank’s balanced salt solution (pH of 6.8; 37°C). Adhered macrophages were treated in triplicate with fresh culture medium RPMI-1640 with FBS and antibiotics (pH of 7.0; 37°C) containing different stimulators as described below. After 24 h more of incubation, the supernatants in triplicates were collected, pooled, and stored at −80°C for cytokine analysis.

This experiment contained 7 individual in vitro assays for testing 7 plant extracts with the same experimental design. The experimental design was a 2 (without or with 1 μg of LPS/mL) × 5 (5 different amounts of each plant extract) factorial arrangement in a randomized complete block design. Therefore, there were a total of 10 treatments for each plant extract. The negative control was the treatment without either plant extract or LPS, and the positive control was the treatment without plant extracts but with LPS. The amounts of anethol, capsicum oleoresin, carvacrol, eugenol, and garlicon tested in this experiment were 0, 25, 50, 100, and 200 μg/mL. The doses of cinnamaldehyde used in this experiment were adjusted to 0, 2.5, 5, 10, and 20 μg/mL according to Chao et al. (2008) and a preliminary experiment. The preliminary experiment found that high doses of cinnamaldehyde were toxic to porcine alveolar macrophages, as 50, 100, and 200 μg/mL of cinnamaldehyde reduced cell viability to 16%, 12%, and 4% respectively. In addition, the amounts of turmeric oleoresin used in this assay were reduced to 0, 2.5, 5, 10, and 20 μg/mL because of the difficulty of dissolving turmeric oleoresin in both DMSO and culture medium.

**Detection of Number of Live Cells**

To determine the toxicity amounts of plant extracts on porcine alveolar macrophages, the 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) assay was used, which measured the metabolic activity of cell cultures with a color reaction catalyzed by mitochondrial enzymes, to detect changes in the number of live cells (Mosmann, 1983). Briefly, after the 24-h incubation of cells in 96-well plates with stimulation and removal of the supernatants as described above, 100 μL of fresh culture medium RPMI-1640 was added to each well. Then 10 μL of 12 mM MTT solution was added to each well. After 4 h of incubation at 37°C, 100 μL of the SDS-HCl (1 mg SDS with 10 mL of 0.01 M HCl) solution was added to each well and mixed thoroughly with a pipette. The plates were incubated at 37°C for 12 h in a humidified chamber. The optical density (OD) was measured at 570 nm with a microtiter plate reader (MTX TC Revelation, DYNEX Technologies, Inc., Chantilly, VA). The background signal inherent to the plates when cells were not present was subtracted from the absorbance obtained from each sample. The OD of the cells in the negative control was taken as the standard and set to 100%. The relative viability was calculated by the following formula: (OD of sample/OD of the control) × 100%. The number of live cells is a function of both viability and proliferation.

**Test of NO**

The Griess assay was used to measure nitrite formed by the spontaneous oxidation of NO (Cho and Chae, 2003). Briefly, 50 μL of cell supernatant was added to each well of the 96-well microplate and incubated with 50 μL of sulfanilamide solution (1% sulfanilamide in 5% phosphoric acid) at room temperature for 5 to 10 min in darkness. Then, 50 μL of 0.1% N-1-naphthylethylenediamine dihydrochloride in water was added to each well and incubated at room temperature for 5 to 10 min in darkness. The OD was measured at 530 nm. Concentrations were calculated from a standard sodium nitrite curve. All samples were analyzed in duplicate. The limit of detection of the kit is 2.5 μM. The intra-assay and interassay coefficients of variation provided by the kit manufacture were lower than 2.7 and 3.4, respectively.

**Measurements of Cytokines**

Protein concentrations of TNF-α, IL-1β, TGF-β, and IL-10 in the cell culture supernatants were measured by ELISA according to the manufacturer’s recommendation. Briefly, standard, control, and samples were added to the wells with coated monoclonal antibody specific for each cytokine. After incubation for 2 h, the unbound substances were washed away, and an enzyme-linked polyclonal antibody specific for the cytokine was added to the wells to sandwich the cytokine immobilized during the first incubation. A further 2 h of incubation was followed by a wash to remove any unbound antibody-enzyme reagent, and then a substrate solution was added to the wells, and color was developed in proportion to the amount of the cytokine bound in the initial step. The color development was stopped by adding the stop solution, and the intensity of the color was measured at 450 nm with the correction wavelength set at 530 nm. Concentrations were calculated from a standard curve. All samples were analyzed in duplicate. The detection limits of the ELISA kit for TNF-α, IL-1β, TGF-β, and IL-10 analyses were 3.7, 10, 4.6, and 1.76 pg/mL, respec-
Plant extracts and porcine alveolar macrophages

RESULTS

Number of Live Cells

Cell viability of porcine alveolar macrophages responded differently to different plant extracts, indicating a range of toxicity. For example, a large dose of capsicum olearin or anethol increased macrophage cell viability (linear, \( P < 0.001 \)) in the absence of LPS stimulation (Table 1). Conversely, cells treated with carvacrol, eugenol, and garlicon showed reduced cell viability (linear, \( P < 0.001 \)). Reduced amounts of cinnamaldehyde and turmeric olearin were used in this experiment as described above. Cinnamaldehyde reduced (linear, \( P < 0.001 \)) the cell viability without LPS stimulation, but still more than 70% of macrophages remained viable. Turmeric olearin also reduced (linear, \( P < 0.001 \)) the cell viability without LPS stimulation, and 20 μg/mL was severely toxic (\( P < 0.001 \)) to macrophages.

The stimulation of LPS inhibited the macrophage cell viability (\( P \leq 0.05 \)), except in the carvacrol and capsicum olearin group. The effects of plant extracts were generally in the same direction as without LPS, with anethol, capsicum olearin, and carvacrol increasing (linear, \( P < 0.001 \)) the number of live cells. However, cinnamaldehyde, eugenol, garlicon, and turmeric olearin did not increase cell viability of macrophages with LPS stimulation.

Table 1. The relative cell viability of porcine alveolar macrophages treated with various concentrations of plant extracts in the absence or presence of 1 μg LPS/mL

<table>
<thead>
<tr>
<th>Item</th>
<th>Without LPS</th>
<th>With LPS</th>
<th>SEM</th>
<th>LPS Level</th>
<th>LPS × amount</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NC(^2)</td>
<td>25 50 100 200</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anethol(^3)–(^5)</td>
<td>100 86 153 114 154</td>
<td>74 69 83 69 99</td>
<td>6.3</td>
<td>&lt;0.001</td>
<td>&lt;0.001 0.002</td>
</tr>
<tr>
<td>Capsicum olearin(^3)</td>
<td>100 110 139 107 159</td>
<td>86 108 132 127 165</td>
<td>6.4</td>
<td>0.818</td>
<td>&lt;0.001 0.012</td>
</tr>
<tr>
<td>Carvacrol(^3)–(^7)</td>
<td>100 101 91 81 —</td>
<td>69 111 101 84 —</td>
<td>11.1</td>
<td>0.751</td>
<td>&lt;0.001 0.047</td>
</tr>
<tr>
<td>Eugenol(^3)–(^4)</td>
<td>100 102 85 72 67</td>
<td>85 77 84 79 71</td>
<td>4.6</td>
<td>0.034</td>
<td>&lt;0.001 0.002</td>
</tr>
<tr>
<td>Garlicon(^3)–(^5)</td>
<td>100 111 91 69 —</td>
<td>85 87 89 78 —</td>
<td>5.3</td>
<td>0.051</td>
<td>&lt;0.001 0.035</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Item</th>
<th>Without LPS</th>
<th>With LPS</th>
<th>SEM</th>
<th>LPS Level</th>
<th>LPS × amount</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NC(^2)</td>
<td>2.5 5 10 20</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cinnamaldehyde(^3)–(^5)</td>
<td>100 83 91 73 70</td>
<td>71 63 73 58 74</td>
<td>5.3</td>
<td>&lt;0.001</td>
<td>&lt;0.001 0.006</td>
</tr>
<tr>
<td>Turmeric olearin(^3)–(^5)</td>
<td>100 87 79 68 —</td>
<td>85 68 67 65 —</td>
<td>4.9</td>
<td>&lt;0.001</td>
<td>&lt;0.001 0.384</td>
</tr>
</tbody>
</table>

\(^1\)LPS = lipopolysaccharide. The unit for the cell viability was percent, and the units for the concentration of plant extracts were micrograms per milliliter.
\(^2\)NC = negative control, with no LPS or plant extracts; PC = positive control, with LPS but no plant extracts.
\(^3\)Linear effect of plant extract level without LPS stimulation, \( P < 0.01 \).
\(^4\)Linear effect of plant extract level with LPS stimulation, \( P < 0.01 \).
\(^5\)Quadratic effect of plant extract level with LPS stimulation, \( P < 0.01 \).
\(^6\)Quadratic effect of plant extract level without LPS stimulation, \( P < 0.01 \).
\(^7\)The high dose of the plant extract was toxic to cells and was removed here.
NO Production

Very low concentrations of NO (less than 2.5 µM) were detected in the supernatants of each treatment in the absence or presence of LPS stimulation. Stimulation by LPS did not induce greater NO secretion from porcine alveolar macrophages (data are not shown).

Proinflammatory Cytokines

The general pattern of TNF-α production in the absence of LPS was an increase at low plant extract concentrations and a progressive decrease as the plant extract concentration increased (Figure 1), although several linear and quadratic effects were not significant. Garlicon differed from this pattern, as there was a linear ($P = 0.012$) effect in TNF-α as its concentration increased. Stimulation by LPS tremendously increased ($P < 0.001$) the secretion of TNF-α from macrophages, but all 7 plant extracts tested dose-dependently inhibited (linear, $P < 0.002$) the secretion of TNF-α from LPS-induced macrophages (Figure 1).

Carvacrol, garlicon, cinnamaldehyde, and turmeric oleoresin increased (linear, $P < 0.01$) the secretion of IL-1β from macrophages in the absence of LPS (Figures 2C, 2E, 2F, and 2G). The inclusion of anethol and capsicum oleoresin also increased (quadratic, $P < 0.01$) the secretion of IL-1β from macrophages in the absence of LPS (Figures 2A and 2B). The LPS sharply increased ($P < 0.001$) the secretion of IL-1β from macrophages. In the presence of LPS, individual plant extract affected secretion of IL-1β differently. The treatments with anethol, capsicum oleoresin, carvacrol, eugenol, garlicon, and cinnamaldehyde suppressed (linear, $P < 0.05$) the secretion of IL-1β from LPS-induced macrophages in a dose-dependent manner (Figures 2A, 2B, 2C, 2D, 2E, and 2F). However, no effect was observed in the turmeric oleoresin treatments (Figure 2G).

Anti-inflammatory Cytokines

Anti-inflammatory cytokines, IL-10 and TGF-β, were analyzed in this experiment, but IL-10 was not detectable in any supernatants of macrophages treated with different stimulators. Compared with the negative control, anethol and capsicum oleoresin decreased (linear, $P < 0.001$) but carvacrol, cinnamaldehyde, eugenol, and garlicon increased (linear, $P < 0.001$) the secretion of TGF-β from macrophages in the absence of LPS (Figures 3C, 3D, 3E, and 3F). The inclusion of turmeric oleoresin increased (quadratic, $P < 0.001$) the concentrations of TGF-β in the supernatant of macrophages without LPS stimulation. The LPS stimulation enhanced ($P < 0.001$) the secretion of TGF-β from macrophages in the presence of LPS, anethol (linear, $P < 0.001$), capsicum oleoresin (linear, $P < 0.001$), carvacrol (linear, $P < 0.001$), and garlicon (quadratic, $P < 0.001$) suppressed the secretion of TGF-β from macrophages (Figures 3A, 3B, 3C, and 3E); however, eugenol (linear, $P < 0.001$), cinnamaldehyde (quadratic, $P < 0.001$), and turmeric oleoresin (linear, $P < 0.001$) increased the secretion (Figures 3D, 3F, and 3G).

DISCUSSION

The present study shows for the first time that the addition of plant extracts alters the secretion of cytokines by porcine cells with or without LPS stimulation. Notably, all 7 plant extracts tested reduced the production of a proinflammatory cytokine by porcine alveolar macrophages stimulated by LPS.

Tumor necrosis factor-α and IL-1β are 2 important proinflammatory cytokines. The mediation of inflammation against infection by these proinflammatory cytokines is beneficial to the host, but overexpression of these cytokines might cause inflammatory diseases (Ferrero-Miliani et al., 2006). Previous studies related to mice, humans, and pigs have reported that LPS stimulated the production of proinflammatory cytokines secreted from macrophages (Lee et al., 2007; Chao et al., 2008; Che et al., 2008). In the present study, it was also found that LPS stimulation sharply increased the secretion of TNF-α and IL-1β from macrophages. The results showed that all 7 plant extracts inhibited the secretion of TNF-α from LPS-induced macrophages in a dose-dependent manner, consistent with previous studies in a human cell line model (Lee et al., 2007; Chao et al., 2008). The TNF-α results indicate that all 7 plant extracts tested here may have potential anti-inflammatory activity.

The modes of action for the anti-inflammatory activity of plant extracts are still not clear, but evidence suggests that these effects are mediated, at least in part, by blocking the transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway (Jobin et al., 1999; Lee et al., 2005; Choi et al., 2007), which is a key regulator of various genes involved in immune and inflammatory responses (Xie et al., 1994). Interestingly, the IL-1β response differed from that of TNF-α in the presence of LPS. The treatments with anethol, capsicum oleoresin, carvacrol, cinnamaldehyde, eugenol, and garlicon significantly suppressed the secretion of both TNF-α and IL-1β from LPS-induced macrophages, but turmeric oleoresin did not affect the secretion of IL-1β from LPS-induced macrophages. Because transcriptional activation of the IL-1β gene depends on NF-κB activation, any impact on this critical event should affect IL-1β transcription in response to LPS (Hiscott et al., 1993). Therefore, the effect of plant extracts on NF-κB activation...
Figure 1. Plant extracts influence the production of tumor necrosis factor-α (TNF-α) from porcine alveolar macrophages in the absence or presence of lipopolysaccharide (LPS). Cells were incubated with various concentrations (0, 25, 50, 100, and 200 μg/mL unless otherwise noted) of each plant extract in the absence or presence of LPS (1 μg/mL) for 24 h. The concentration of TNF-α secreted by porcine alveolar macrophages treated with (A) anethol, (B) capsaicin oleoresin, (C) carvacrol, (D) eugenol, (E) garlic, (F) cinnamaldehyde (0, 2.5, 5, 10, and 20 μg/mL), and (G) turmeric oleoresin (0, 2.5, 5, 10, and 20 μg/mL) is presented as picograms per milliliter. The largest dose of (C), (E), and (G) were toxic to cells and were removed here. The results were means of values from 6 pigs. For anethol (A), LPS: \( P < 0.001 \); level, \( P = 0.084 \); interaction: \( P = 0.084 \). For garlic (E), LPS: \( P < 0.001 \); level, \( P = 0.004 \); interaction: \( P = 0.004 \). For turmeric oleoresin (G), LPS: \( P < 0.001 \); level, \( P = 0.172 \); interaction, \( P = 0.197 \). For all other plant extracts, LPS: \( P < 0.001 \); level: \( P < 0.001 \); interaction: \( P < 0.001 \).
Figure 2. Plant extracts influence the production of IL-1β from porcine alveolar macrophage in the absence or presence of lipopolysaccharide (LPS). Cells were incubated with various concentrations (0, 25, 50, 100, and 200 μg/mL unless otherwise noted) of each plant extract in the absence or presence of LPS (1 μg/mL) for 24 h. The concentration of IL-1β secreted by porcine alveolar macrophages treated with (A) anethol, (B) capsicum oleoresin, (C) carvacrol, (D) eugenol, (E) garlicon, (F) cinnamaldehyde (0, 2.5, 5, 10, and 20 μg/mL), and (G) turmeric oleoresin (0, 2.5, 5, 10, and 20 μg/mL) is presented as picograms per milliliter. The largest dose of (C), (E), and (G) were toxic to cells and were removed here. The results were means of values from 6 pigs. For anethol (A), LPS: $P < 0.001$; level: $P < 0.001$; interaction: $P = 0.016$. For capsicum oleoresin (B), LPS: $P < 0.001$; level: $P = 0.147$; interaction: $P = 0.151$. For carvacrol (C), LPS: $P < 0.001$; level: $P < 0.001$; interaction: $P = 0.146$. For turmeric oleoresin (G), LPS: $P < 0.001$; level: $P = 0.820$; interaction: $P = 0.830$. For all other plant extracts, LPS: $P < 0.001$; level: $P < 0.001$; interaction: $P < 0.001$. 
Figure 3. Plant extracts influence the production of transforming growth factor-β (TGF-β) from porcine alveolar macrophages in the absence or presence of lipopolysaccharide (LPS). Cells were incubated with various concentrations (0, 25, 50, 100, and 200 μg/mL unless otherwise noted) of each plant extract in the absence or presence of LPS (1 μg/mL) for 24 h. The concentration of TGF-β secreted by porcine alveolar macrophages treated with (A) anethol, (B) capsicum oleoresin, (C) carvacrol, (D) eugenol, (E) garlicon, (F) cinnamaldehyde (0, 2.5, 5, 10, and 20 μg/mL), and (G) turmeric oleoresin (0, 2.5, 5, 10, and 20 μg/mL) is presented as picograms per milliliter. The largest dose of (C), (E), and (G) were toxic to cells and were removed here. The results were means of values from 6 pigs. For anethol (A), cinnamaldehyde (F), and turmeric oleoresin (G), LPS: P < 0.001; level: P < 0.001; interaction: P < 0.001. For capsicum oleoresin (B), carvacrol (C), eugenol (D), and garlicon (E), LPS: P < 0.001; interaction: P < 0.001.
should be assessed in the future, particularly because we found different results for IL-1β than for TNF-α, which is transcriptionally regulated not only by NF-κB but also by other pathways (Collart et al., 1990; Ndengele et al., 2000). The high dose of turmeric oleoresin (20 μg/mL) in the presence of LPS reduced both the number of live macrophages and cytokine secretion, suggesting the primary effect may be cytotoxicity rather than suppression of cytokine production. On the other hand, in the absence of LPS, anethol, capsicum oleoresin, cinnamaldehyde, garlicon, and turmeric oleoresin stimulated the secretion of TNF-α, IL-1β, or both from macrophages, which indicates that the plant extracts may have the potential ability to enhance immune responses in normal conditions.

One of the important anti-inflammatory cytokines found in the immune response is IL-10. It can suppress the secretion of proinflammatory cytokines from macrophages through several different ways (Opal and DePalo, 2000). The major part of IL-10 synthesis is stimulated by proinflammatory cytokines, such as TNF-α, and also requires the activation of other protein kinases or pathways (Wandworanun and Strober, 1993; Meisel et al., 1996). However, the present study indicates that macrophages failed to synthesize significant amounts of IL-10 in response to LPS stimulation. The results are consistent with those of Thomassen et al. (1996), Salez et al. (2000), and Daniels et al. (2011), who found a lack of IL-10 synthesis by human, murine, or porcine alveolar macrophages upon LPS stimulation. This failure of IL-10 synthesis was not due to an absence of porcine alveolar macrophage activation or to a lack of proinflammatory cytokines because TNF-α and IL-1β were detected as expected. The underlying mechanism accounting for the absence of IL-10 synthesis is not clear. Salez et al. (2000) suggested that the IL-10 protein expression is regulated at the pretranscriptional level, and some unknown pulmonary environmental factors might suppress IL-10 mRNA expression by alveolar macrophages.

Transforming growth factor-β is another interesting cytokine involved in the immune response. Like many cytokines, TGF-β has both immune-suppressive and immune-enhancing activities (Opal and DePalo, 2000). As an anti-inflammatory cytokine, TGF-β can suppress the proliferation and differentiation of T and B cells and deactivate monocyte/macrophage in a manner similar to IL-10 (Letterio and Roberts, 1997). However, as a proinflammatory cytokine, TGF-β in the presence of different cytokines can drive the differentiation of diverse T helper cells, which promote further tissue inflammation (Sanjabi et al., 2009). In the present study, the treatments with the different plant extracts tested here showed different effects on the secretion of TGF-β from macrophages in the presence of LPS and show little relationship of secretion of TGF-β to that of proinflammatory cytokines.

The viability test using the MTT assay was performed to make sure the influence of plant extracts on the inflammatory mediators secreted from porcine alveolar macrophages resulted from mechanisms other than direct killing of cells. The high level (200 μg/mL) of carvacrol and garlicon were cytotoxic to macrophages, and very low amounts of cinnamaldehyde and turmeric oleoresin significantly inhibited cell viability of macrophages. However, anethol and capsicum oleoresin increased cell viability of macrophages. These results indicated different effects of different plant extracts on cell viability of macrophages. On the basis of the MTT results, the data for the greatest amount of carvacrol (200 μg/mL), garlicon (200 μg/mL), and turmeric oleoresin (20 μg/mL) were removed here because of the cytotoxic effect on macrophages. The MTT results in the LPS group also indicated that the inhibitory effects of plant extracts on the response to this proinflammatory mediator probably resulted from mechanisms other than direct killing of cells.

Nitric oxide is a very important molecule involved in a wide range of physiologic and pathologic processes in mammalian systems, and its production by macrophages is fundamental for immune defense (MacMicking et al., 1997). Previous studies from Lee et al. (2002) and Li et al. (2006) reported cinnamaldehyde and eugenol suppressed NO production from LPS-treated murine macrophages. However, in the present study, the stimulation by 1 μg of LPS/mL did not affect the NO production of macrophages, confirming the previous findings of Pampusch et al. (1998) and Zelnickova et al. (2008), who also failed to induce NO production from porcine alveolar macrophages with LPS stimulation. The LPS stimulation increased the secretion of proinflammatory cytokines, indicating that the inability of macrophages to produce NO was not caused by nonreactivity to stimulation with LPS. The fundamental differences among species in the abilities of macrophages to produce NO are not clear. In the present study, there were detectable effects of specific plant extracts on NO production from macrophages, but they were all small in magnitude and therefore not of clear importance.

In conclusion, the present results show the ability of all plant extracts used in this study to inhibit LPS-induced production of the proinflammatory cytokine TNF-α by porcine alveolar macrophages. In addition, several plant extracts can also suppress IL-1β secretion. These results indicate that all of these plant extracts may have potent anti-inflammatory effects. Especially, carvacrol, cinnamaldehyde, eugenol, and garlicon might be the more powerful candidates because they block the secretion of both of the proinflammatory cytokines measured, TNF-α and IL-1β. These observations require verification in vivo.
LITERATURE CITED
