

Rosmarinic Acid in *Prunella vulgaris* Ethanol Extract Inhibits Lipopolysaccharide-Induced Prostaglandin E2 and Nitric Oxide in RAW 264.7 Mouse Macrophages

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Prunella vulgaris has been used therapeutically for inflammation-related conditions for centuries, but systematic studies of its anti-inflammatory activity are lacking and no specific active components have been identified. In this study, water and ethanol extracts of four *P. vulgaris* accessions were applied to RAW 264.7 mouse macrophages, and the ethanol extracts significantly inhibited lipopolysaccharide (LPS)-stimulated prostaglandin E2 (PGE2) and nitric oxide (NO) production at 30 μg/mL without affecting cell viability. Extracts from different accessions of *P. vulgaris* were screened for anti-inflammatory activity to identify accessions with the greatest activity. The inhibition of PGE2 and NO production by selected extracts was dose-dependent, with significant effects seen at concentrations as low as 10 μg/mL. Fractionation of ethanol extracts from the active accession, Ames 27664, suggested fractions 3 and 5 as possible major contributors to the overall activity. Rosmarinic acid (RA) content in *P. vulgaris* was found to independently inhibit inflammatory response, but it only partially explained the extracts' activity. LPS-induced cyclooxygenase-2 (COX-2) and nitric oxide synthase (iNOS) protein expression were both attenuated by *P. vulgaris* ethanol extracts, whereas RA inhibited only COX-2 expression.

KEYWORDS: *Prunella vulgaris*; extracts; fractionation; prostaglandin E2; nitric oxide; rosmarinic acid; anti-inflammatory

INTRODUCTION

Prunella vulgaris (Lamiaceae), commonly called self-heal, is a perennial herb widely distributed in Asia and Europe. In Europe, *P. vulgaris* has been a popular traditional remedy since the 17th century for several medical conditions including mild fever, sore throat, and external wound healing (1). In China, *P. vulgaris* is called “Xia Ku Cao” and has a long history in therapeutic use as an antipyretic and, more recently, for antikeratitis purposes (2). In South Korea, *P. vulgaris* is applied to patients with goiter, dermatitis, and skin allergy (3). Despite its wide use for health purposes, only a few scientific studies have addressed the health-promoting claims of *P. vulgaris*. Aqueous extracts of *P. vulgaris*, rich in carbohydrates, were reported to have anticarcinogenic, counter-UV damage, immune-regulatory, and antiviral effects (4–6). In addition, increasing evidence suggests that extracts prepared with organic solvents, such as ethanol and methanol, possess antiestrogenic, anti-inflammatory, and anti-oxidative properties (7–9).

Although most of the bioactivities seen in *P. vulgaris* water extracts are attributed to polysaccharide compounds, no specific component in these extracts has been associated with anti-inflammatory activity (6). On the other hand, a polyphenol-rich aqueous ethanolic extract (30% v/v) contains two known constituents with anti-inflammatory activity, namely, rosmarinic acid (RA) and ursolic acid (UA) (8, 10). Information regarding the relative abundance of possible anti-inflammatory compounds in different populations of *P. vulgaris* is not available, and direct comparison of activity among different accessions has not been reported.

Inflammation is critical in recruiting immune cells and molecules to the site of infection for defense. However, various kinds of tissue damage and pathological consequences ensue when “sterile inflammation”, such as obesity and self-immune diseases, occurs or infection-induced inflammation becomes chronic. Among participating cells, macrophages play a central role in organizing the release of inflammation mediators, including prostaglandin E2 (PGE2), nitric oxide (NO), and cytokines that promote host protection, as well as causing pathological consequences such as tissue edema and abnormal histological change (8, 11). The RAW

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264.7 mouse macrophage cell line is widely used for studies of inflammation, due to its reproducible response to lipopolysaccharide (LPS), mediated by toll-like receptor 4 (TLR4) (12). Our previous research on *Hypericum* and *Echinacea* species successfully employed this model to study the effects of these botanical materials on cyclooxygenase-2 (COX-2) regulated PGE2 production and upstream intracellular events (13, 14). Other research with these cells indicates a well-defined nitric oxide induction by LPS through inducible nitric oxide synthase (iNOS) (15). Therefore, we used this model to investigate the effect of *P. vulgaris* extracts on these two major inflammatory mediators.

The main purpose of the current study was to compare anti-inflammatory activity of water and ethanol extracts of *P. vulgaris* in LPS-stimulated RAW 264.7 mouse macrophages and to assess any differences in activity among different *P. vulgaris* accessions. Fractionation of active extracts allowed us to explore possible bioactive compounds. At the same time, parallel comparison of pure compounds and extract activities revealed the contribution of selected compounds.

MATERIALS AND METHODS

Plant Materials. Vegetative samples of *P. vulgaris* were acquired from the North Central Regional Plant Introduction Station (NCRPIS) of the U.S. Department of Agriculture, Agricultural Research Service (USDA/ARS), Ames, IA. This study included accessions Ames 27664, 27665, and 27666 from North Carolina, Ames 27748 and 28436 (PI 656842) from Missouri, and Ames 28353 (PI 656839), 28354 (PI 656840), 28355, 28356, 28357 (PI 656841), 28358, and 28359 from Iowa. Further detailed information about the origins of these accessions is available from the Germplasm Resources Information Network (GRIN) database at http://www.ars-grin.gov/npgs/acc/acc_queries.html.

Seeds from original samples of all these accessions were germinated at 25 °C in Petri plates and then transferred to greenhouse flats held at 20–25 °C. In April 2006, seedlings of Ames 27664, 27665, 27666, and 27748 were transplanted to the field at 2 months of age, and each individual accession was isolated within control-pollination cages. In October 2007, above-ground portions of these accessions were harvested and air-dried at ~40 °C for 1 week. Dried samples were then ground in a Wiley mill and stored at –20 °C under nitrogen until extraction.

In May 2007, 2-month-old seedlings of the remaining accessions were transplanted to the same field with the same isolation procedure. In July 2008, all accessions in the field, except for Ames 27666, were harvested, air-dried, ground, and stored, as noted above.

The taxonomic identity of each accession was confirmed at the time of flowering. Seed samples for each accession are conserved and distributed by the NCRPIS, and corresponding voucher specimens are held at the Ada Hayden Herbarium, Iowa State University.

Sterilization Technique. All glassware was heated at 200 °C for 2 h to destroy endotoxin, whereas other supplies were purchased sterile. Random samples of supernatant and cell pellet were chosen from cell culture in selected experiments for mycoplasma screening with a MycoProbe mycoplasma detection kit (R&D Systems, Minneapolis, MN), and no contamination was found. Water, ethanol, and DMSO solvents, along with extraction and fractionation products, were tested for endotoxin by using a Lymulus Amebocyte Lysate Test (Bio Whittaker, Walkersville, MD) (14). The endotoxin levels ranged from undetectable to 0.000658 EU/mL for all extracts and ethanol fractions, well below the 5 EU/mL threshold for significant stimulation of RAW 264.7 macrophages (13). Endotoxin levels of water fractions, ranging from 0.0001 to 0.0044 EU/mL, were higher than those of extracts and ethanol fractions.

Rosmarinic Acid and Ursolic Acid. Rosmarinic acid and ursolic acid at 90–100 and 95% purity, respectively (as graded by the manufacturer, Fisher Scientific, Hanover Park, IL), were dissolved in dimethyl sulfoxide (DMSO) to 100 and 50 mM stock concentration, respectively, and stored at –20 °C.

Extraction and Fractionation of *P. vulgaris*. *Ethanol Extraction.* Six grams of dried *P. vulgaris* ground sample was extracted with 500 mL of 95% ethanol via Soxhlet for 6 h. The extract was filtered and then dried by

Table 1. Solvent Gradient for Ethanol Extract Fractionation^a

solvent	duration					
	30 min	10 min	40 min	10 min	40 min	10 min
A	90–70%	70–60%	60–20%	20–0%	0%	0–90%
B	10–30%	30–40%	40–80%	80–100%	100%	100–10%

^a Solvent A is water with 0.1% acetic acid. Solvent B is acetonitrile.

rotary evaporation at < 40 °C followed by lyophilization. The extract was then dissolved in the minimal amount of DMSO that completely dissolved the residue and stored at –20 °C.

Water extraction. Six grams of dried *P. vulgaris* ground sample was extracted with 100 mL of boiling endotoxin-free water. The plant material was steeped with stirring for 1 h and then filtered through a G6 glass fiber circle (Fisher Scientific, Hanover Park, IL) in a Buchner funnel. The filtrate was centrifuged at 10000g for 20 min to remove additional particulates. Then, the extract was lyophilized, weighed, and redissolved with the minimal volume of endotoxin-free water that dissolved the residue and stored at –20 °C.

Size Exclusion Chromatography Fractionation of the Water Extract. Two grams of dry *P. vulgaris* Ames 27664 water extract residue, dissolved in 10 mL of endotoxin-free water, was loaded onto a 2.5 × 75 cm Sephacryl 100HR column. Elution was with endotoxin-free water, and the eluent was collected in 10 mL individual fractions over the following 72 h until a combined volume of 2 L was recovered. The absorbance at 210 nm was measured for all tubes. Nine peaks were reserved, according to which these fractions were pooled into eight fractions and concentrated by lyophilization, after the last two peaks had pooled in the same fraction due to their low yield. The residues after lyophilization were then completely dissolved in the same volume of water, and all fractions were stored at –20 °C.

Semipreparative HPLC Fractionation of the Ethanol Extract. One hundred milligrams of dry *P. vulgaris* Ames 27664 ethanol extract residue, dissolved in 0.5 mL of 60% ethanol, was loaded onto an YMC-pack ODS-AM 250 × 10 mm C₁₈ column (YMC, Kyoto, Japan). The HPLC system used was a Beckman-Coulter System Gold with a 126 solvent module, a model 508 autosampler, and a model 168 detector. Solvents were endotoxin-free water containing 0.1% acetic acid as A and acetonitrile as B, following the gradient shown in Table 1. On the basis of the absorbance peaks at 210 nm, 2 mL aliquots of fractions from the ethanol extract were pooled into seven fractions, which were concentrated by lyophilization and later dissolved with the same amount of DMSO for storage at –20 °C.

Cell Culture and Treatment with Plant Materials. RAW 264.7 mouse macrophage cells (American Type Culture Collection, Manassas, VA) were cultured as described by Hammer et al. (13) in high-glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 10% sodium bicarbonate, and 100 IU/mL penicillin/streptomycin (all from Invitrogen, Carlsbad, CA). The cells were incubated at 37 °C under 5% CO₂ and grew to 80% confluency before passaging or plating. Cells were plated in 24- or 48-well plates or on Petri dishes (Corning, Lowell, MA) for treatment as described by Hammer et al. (13). When the plated cells reached 80% (for 8 h treatment) or 60% (for 24 h treatment) confluency, the stock of *P. vulgaris* extracts and fractions, or pure compounds, was diluted to 1000× treatment concentration with vehicle solvent and then added to media at 0.1%. Treatments in 500 μL of media were applied to macrophages in 24-well plates for PGE2 and NO assay. For cytotoxicity assay, 300 μL of treatment was applied to the 48-well plates. For Western blotting, treatments in 10 mL of media were added to the Petri dishes. Except for the cytotoxicity assay, which was conducted without induction, each treatment was administered with and without stimulation by 1 μg/mL LPS (*Escherichia coli* 02B:B6) (Sigma, St. Louis, MO). Three replicates were included on three individual plates. Aside from media control and DMSO vehicle control at 0.1% v/v, a 10 μM (100 μM for Western blotting) quercetin (Sigma) treatment was also included as a positive control in each experiment to validate each assay because of its proven anti-inflammatory activity as well as its presence in *P. vulgaris* ethanol extract at a trace amount (16). Two additional strategies were also attempted for PGE2 assay, one using a 30 min pretreatment with extracts before LPS induction and the other using a 2 h LPS induction before administration of plant extracts.

Cell Viability Measurement. Cytotoxicity was assayed on all extracts, fractions, and pure compounds following a protocol modified from that of Schmitt et al. (17). Ursolic acid, a known cytotoxic constituent of *P. vulgaris* plant material that accounts for 0.05–0.2% of dry matter (18), was used as the positive control at 10 and 30 μM . The maximal treatment dose used in the bioactivity assays was applied to cells for 24 h in 48-well plates before being replaced by 200 μL of fresh media and 30 μL of Celltiter96 Aqueous One Solution Cell Proliferation Assay solution (Promega, Madison, WI). After a 195 min incubation, 200 μL of metabolized dye product was transferred from each well to a 96-well plate, and absorbance was read by a plate reader at 562 nm. Percentages of viability compared to media + DMSO control were determined for all treatments.

Prostaglandin E2 and Nitric Oxide Measurement. Treatment lasted 8 h for PGE2 assay and 24 h for NO assay before all supernatant in each well was collected on ice for future assays (19). After collection, the supernatant was kept at $-80\text{ }^\circ\text{C}$ before the PGE2 assay was conducted. Samples were diluted (1:15) and analyzed with Biotrek PGE2 enzyme immune assay (EIA) (GE Healthcare, Piscataway, NJ) according to the manufacturer's protocol. PGE2 concentrations in the supernatant samples were determined by comparison with a standard curve.

For the NO assay, the supernatant was collected after 24 h of treatment and stored at $4\text{ }^\circ\text{C}$ before analysis. Griess reagent (Promega) was employed to indirectly measure NO production in cell culture by measuring nitrite concentration (20). Fifty microliters of supernatant sample or a series of standards was mixed with 50 μL of sulfanilamide solution (1% sulfanilamide in 5% phosphoric acid) in a 96-well plate for a 7 min incubation on a rocker in the dark. Then 50 μL of 1% *N*-1-naphthylethylenediamine dihydrochloride (dissolved in water) was added followed by another 7 min incubation. Optical absorbance was measured at 562 nm, and nitrite concentrations in supernatant samples were obtained with reference to a standard curve.

Quantification of Rosmarinic Acid and Ursolic Acid. Identification and quantification of rosmarinic acid in *P. vulgaris* extract were performed with a Beckman Coulter System Gold Nouveau HPLC coupled to Beckman System Gold 168 UV-vis diode array detector (PDA) controlled by 32karat software (version 5.0) with a Supelcosil LC-18 (250 \times 4.6 mm, 5 μm) column (Sigma). The solvent system was 5% acetic acid in water as A and 25% acetonitrile plus 5% acetic acid as B at a flow rate of 1.0 mL/min. The following gradient was used: 40% B/60% A to 70% B/30% A for 10 min, then to 100% B for 1 min, and finally to 40% B/60% A for 12 min. Rosmarinic acid concentrations were determined with UV absorbance at 326 nm in comparison with standard compound (Cayman Chemicals, Ann Arbor, MI). A reverse phase analytical YMC-pack ODS C₁₈ (250 \times 4.6 mm, 5 μm) column (YMC) was used under room temperature for ursolic acid identification and qualification. The mobile phase used for ursolic acid analysis was 1.25% phosphoric acid and acetonitrile at 0.5 mL/min. The gradient used was 15% acetonitrile at 0 min, increased to 84% over 15 min, held for 40 min, and finally decreased to 15% at 65 min. Ursolic acid concentrations were determined with UV absorbance at 210 nm in comparison with standard compound (Sigma).

Enzyme Expression Measurement. For COX-1 and COX-2 measurement, cell lysate was acquired after an 8 h treatment as previously described (21), followed by SDS-PAGE separation and ECL detection, whereas iNOS samples were collected after a 24 h treatment. Mouse monoclonal primary antibodies against COX-1 (sc-19998), iNOS (sc-7271), COX-2 (sc-19999), and α -tubulin (sc-8035) (Santa Cruz Biotechnology, Santa Cruz, CA) were diluted to 1:1000, 1:600, 1:1200, and 1:2000 individually in 5% powdered milk in Tris buffer saline with 0.1% Tween-20. Arbitrary densities after normalization of four replicate blots were archived by Quantity One program (Bio-Rad, Hercules, CA).

Statistical Analysis. For the cytotoxicity assay, three measurements of each treatment were collected in each of three plates and averaged within plates. PGE2 and NO activities for treatments in each experiment were measured on three plates. For other experiments, a randomized complete block ANOVA was conducted on log-transformed PGE2 concentration, NO concentration, and cell viability values, with the plate as fixed block to identify treatment effect. For each assay, a control treatment was included for media and vehicle (water or DMSO) with and without LPS stimulation. All treatments and the water vehicle control were compared against the media + DMSO vehicle control, and the averages

and standard errors as percentage of vehicle control for each treatment were reported. Multiple comparisons among treatments for Western blotting data were conducted with a pairwise Student *t* test after a significant difference was found among treatments by ANOVA (SAS 9.0 SAS Institute Inc., Cary, NC).

RESULTS

Cytotoxicity of *P. vulgaris* Extracts and Fractions on RAW 264.7 Macrophages. All water and ethanol extracts, from all accessions included in this study, were screened for cytotoxicity at 30 $\mu\text{g}/\text{mL}$, the maximal concentration used in this study. In addition, fractions from certain extracts were also screened at the concentrations used in activity assays. Cytotoxicity was not observed with RAW 264.7 macrophages treated with any extract or fraction, whereas the ursolic acid, as a positive cytotoxic control, caused 50% reduction of cell viability ($p < 0.01$) at 30 μM (150-fold the concentration found in *P. vulgaris* extracts).

Inhibition of LPS-Induced PGE2 and NO Production by *P. vulgaris* Extracts. Both water and ethanol extracts from all four 2007-harvested *P. vulgaris* accessions (Ames 27664, 27665, 27666, and 27748) were included in the initial screening for inhibition of PGE2 and NO production at 30 $\mu\text{g}/\text{mL}$ concentration, the highest concentration achievable with all extracts. As shown in **Figure 1**, ethanol extracts from all accessions except Ames 27748 significantly inhibited LPS-induced PGE2 production as compared to the DMSO vehicle control treatment. On the contrary, water extracts did not exert significant inhibition of PGE2 production at the same concentration. Ethanol extracts from Ames 27664, 27665, and 27666 inhibited LPS-stimulated PGE2 by 36, 23, and 25%, respectively. LPS-induced NO production was significantly inhibited by all extracts except for the water extract from Ames 27748. Water extracts from Ames 27664, 27665, and 27666 significantly decreased NO levels by 10–15%, which were less potent than their ethanol-extracted counterparts, which inhibited NO by 24–26%. Although Ames 27748 water extract had no significant effect on NO production, ethanol extract from this accession decreased NO by 69% as compared to vehicle control. Different treatment strategies, including pretreatment with extracts or LPS, as described under Materials and Methods, did not change the observed effect (data not shown). No change in either baseline PGE2 or NO production was seen when RAW 264.7 macrophages were treated with extracts without LPS stimulation (data not shown).

Difference of Inhibition Potency among Ethanol Extracts from Different *P. vulgaris* Accessions. Ethanol extracts from all accessions harvested in 2008 were applied to cell culture at 30 $\mu\text{g}/\text{mL}$ concentration. As shown in **Table 2**, in comparison to the DMSO vehicle control, accessions Ames 27664, 28358, 28357, and 28355 significantly reduced LPS-stimulated PGE2 production, and all extracts significantly decreased LPS-induced NO. Relatively greater overall inhibition of LPS-induced PGE2 and NO release was observed on extracts from accessions Ames 27664, 28358, 28357, and 28355.

Dose-Response Relationship of the Inhibition on PGE2 and NO Production by Selected *P. vulgaris* Ethanol Extracts. Because the ethanol extract from accession Ames 27664 harvested in 2007 had the greatest inhibitory activity on LPS-induced PGE2 and NO release among all accessions from both years tested, the dose-response relationship for its activity was evaluated. Four different doses were applied to cells: 30, 20, 15, and 7.5 $\mu\text{g}/\text{mL}$. The resulting effect on LPS-induced inflammatory response is shown in **Figure 2**, indicating the inhibition on LPS-induced PGE2 and NO production followed a dose-dependent pattern with stronger inhibition accompanied at higher concentration.

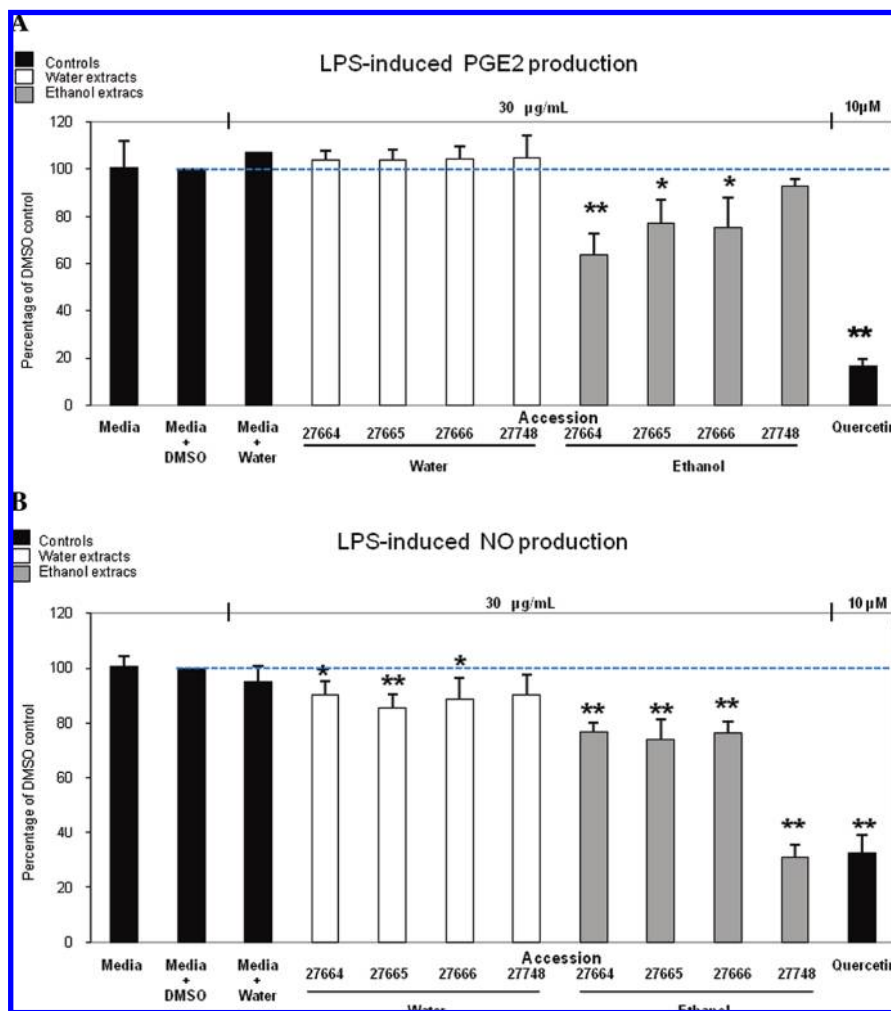


Figure 1. RAW 264.7 macrophages were treated with water and ethanol extracts from four accessions of *P. vulgaris* harvested in 2007 at 30 µg/mL with or without 1 µg/mL LPS induction. The resulting LPS-stimulated PGE2 (A) and NO productions (B) are presented as percentage of vehicle control media + DMSO treatment in mean ± SEM ($N = 3$). The 100% levels of PGE2 and NO productions were 3.94 ± 0.37 ng/mL and 19.4 ± 1.21 µM, respectively. Statistically significant differences compared to the vehicle control are marked with asterisks (*, $p < 0.05$; **, $p < 0.01$). Quercetin was used as a positive control at 10 µM concentration. No difference was found in PGE2 and NO production among treatments without LPS induction, which is not shown.

Table 2. Inhibition of PGE2 and NO Production by Ethanol Extracts from Accessions in 2008 Harvest^a

NCRPIS accession (Ames no.)	% PGE2 inhibition (± SEM)	% NO inhibition (± SEM)
27664	18 ± 3*	19 ± 3**
28353	13 ± 4	27 ± 1**
28354	12 ± 3	18 ± 5**
28355	26 ± 3**	24 ± 1**
28356	11 ± 8	27 ± 3**
28357	16 ± 4*	34 ± 6**
28358	19 ± 6*	33 ± 3**
28359	11 ± 1	27 ± 1**
28436	11 ± 8	27 ± 7**

^a Percentage inhibition of LPS-induced PGE2 and NO released by RAW 264.7 macrophages compared to media + DMSO vehicle control is shown as mean ± SEM (%). Significant reduction is highlighted with bold numbers as well as labeled with asterisks (*, $p < 0.05$; **, $p < 0.01$; $N = 3$).

Ethanol extracts of accessions Ames 27664, 28358, and 28355 from the 2008 harvest were also applied to cell culture at serial doses to establish the dose–response relationship. For each extract, administration concentrations were 30, 10, and 5 µg/mL. As shown in **Figure 3**, all three extracts demonstrated a dose-dependent activity in reducing PGE2 and NO production,

although the reduction was not statistically significant at lower concentrations. Consistent with the initial screening, these extracts significantly inhibited both LPS-induced PGE2 and NO release at the starting concentration of 30 µg/mL. When the dose was decreased, the inhibition was weakened, as only accessions Ames 27664 and 28355 showed significant effect at 10 µg/mL on PGE2 and NO production, respectively.

Contribution of Fractions to the PGE2 and NO Inhibition by *P. vulgaris* Extract. Both water and ethanol extracts from accession Ames 27664 (2007 harvest) were fractionated and screened for activity. As panels A and B of **Figure 4** show, at the concentrations proportional to their yield from the extract, fractions 1, 3, and 5 significantly inhibited LPS-induced PGE2 production, whereas NO level was reduced by all fractions. When the dose was normalized to 20 µg/mL, only fraction 3 significantly reduced both PGE2 and NO production by 28 and 8%, respectively. Fraction 5 also significantly alleviated NO release by 11%, as indicated by **Figure 4C,D**. In contrast, fractions from the water extract of the same plant material did not exert any significant inhibition on LPS-stimulated PGE2 or NO release (data not shown). The baseline PGE2 and NO productions without LPS induction were significantly elevated compared to control and other treatments. To clarify whether the ethanol extract had

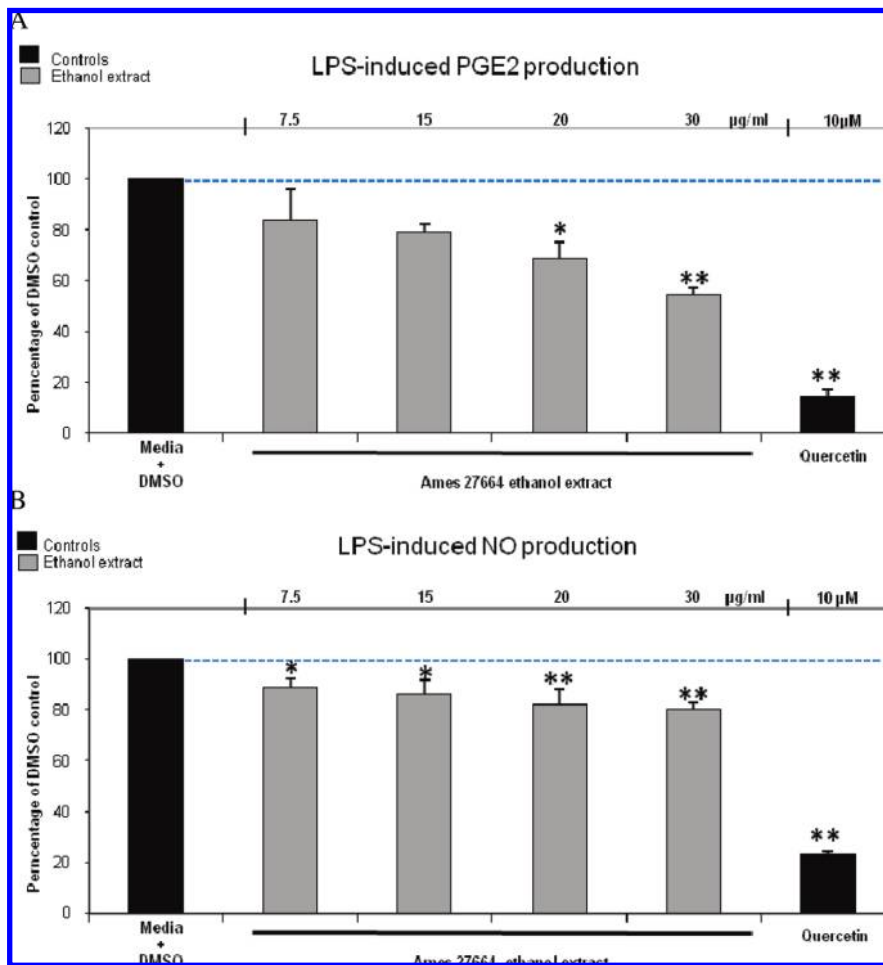


Figure 2. Ethanol extracts from accessions Ames 27664 (2007 harvest) were applied to RAW 264.7 macrophages at 7.5, 15, 20, and 30 $\mu\text{g/mL}$ with or without 1 $\mu\text{g/mL}$ LPS induction. PGE2 (**A**) and NO (**B**) levels as percentage of media + DMSO vehicle control are shown as mean \pm SEM ($N=3$). The 100% levels of PGE2 and NO were 4.84 ± 0.30 ng/mL and 10.5 ± 0.97 μM , respectively. Statistically significant differences compared to the vehicle control are marked with asterisks (*, $p < 0.05$; **, $p < 0.01$). Quercetin was used as a positive control at 10 μM concentration. No difference was found in PGE2 and NO production without LPS stimulation among treatments, which is not shown.

active components that were also present in the water extract, a sequential extraction with water was performed on Ames 27664 ethanol extract. The re-extracted product was tested for activity against LPS-induced NO release along with the ethanol extract. Although the ethanol extract significantly reduced NO production by 22%, the sequential ethanol then water extraction product had no activity compared to the vehicle control.

Rosmarinic Acid and Ursolic Acid in *P. vulgaris* Ethanol Extracts and Fractions. Because RA is a proven anti-inflammatory and antioxidative polyphenol carboxylic component of *P. vulgaris* (8, 22), its abundance was quantified (Table 3). The overall mean \pm SE RA content for all accessions was 2.1 ± 0.3 μM in 30 $\mu\text{g/mL}$ extract. Extracts from Ames 27748 and 27664 (2007 and 2008 harvests) contained significantly more RA than did the others, whereas those from Ames 28356 and 28359 had the lowest RA concentrations. Fractions from Ames 27664 ethanol extracts also contained rosmarinic acid (Table 4). Fractions 5 and 7 had significantly more RA at concentrations in proportion to their yield. When normalized to the same fraction concentration of 20 $\mu\text{g/mL}$, only fraction 5 contained a higher amount of RA. UA concentration is very low in *P. vulgaris* ethanol extract as we detected 0.2 μM in 30 $\mu\text{g/mL}$ Ames 27664 extract and none in any of its fractions.

Inhibition of PGE2 and NO by Rosmarinic Acid in *P. vulgaris* Ethanol Extracts. The effects of pure RA, ethanol extracts, and

extracts enriched with RA were compared on LPS-induced PGE2 and NO production. Pure RA compound was applied to cell culture at 2.67 μM , the concentration found in Ames 27664 ethanol extract from the 2007 harvest. The enrichment dose was also 2.67 μM in order to allow us to assess whether an additive or synergistic effect existed between RA and other components of the extracts. As displayed in Figure 5, RA alone significantly inhibited both LPS-induced PGE2 and NO production by 15 and 17%, respectively. Ames 27664 ethanol extract had a significantly stronger inhibition of 31 and 20%, whereas the "RA-enriched" ethanol extract from the same accession inhibited the two inflammation mediators by as much as 39 and 29%. RA enrichment significantly enhanced the potency of the extract in an additive fashion; no interaction between RA and whole extracts was observed ($p = 0.85$) when analyzed with a 2×4 two-way ANOVA (RA effect and extracts effect).

COX-2 and iNOS Expression with *P. vulgaris* Ethanol Extracts and Rosmarinic Acid Treatment. After 8 h of treatment with ethanol extracts from Ames 27664 harvested in 2007 and 2008, as well as with pure RA, COX-2 expression in RAW 264.7 macrophages was assayed by Western blot. As shown in Figure 6, *P. vulgaris* ethanol extracts and pure RA significantly suppressed LPS-stimulated COX-2 expression as compared to media + DMSO vehicle control. There was no significant difference in treatment effect on COX-2 expression except that the 2007 extract

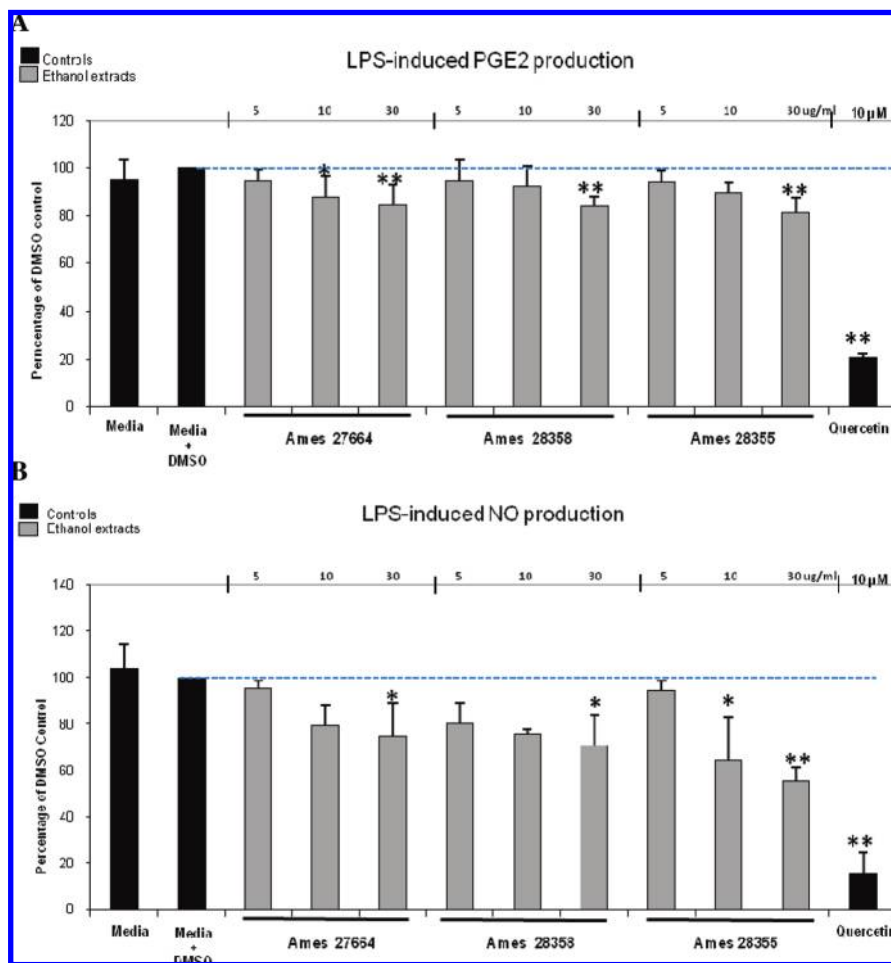


Figure 3. Ethanol extracts from accessions Ames 27664, 28358, and 28355 (2008 harvest) were applied to RAW 264.7 cells at 5, 10, and 30 $\mu\text{g}/\text{mL}$ with or without 1 $\mu\text{g}/\text{mL}$ LPS induction. PGE2 (A) and NO (B) production as percentage of media + DMSO vehicle control is shown as mean \pm SEM ($N = 3$). The 100% levels of PGE2 and NO were 3.86 ± 0.40 ng/mL and 7.7 ± 0.98 μM , respectively. Statistically significant differences compared to the vehicle control are marked with asterisks (*, $p < 0.05$; **, $p < 0.01$). Quercetin was used as a positive control at 10 μM concentration. No difference was found in PGE2 and NO production without LPS stimulation among treatments, which is not shown.

was more effective than those from 2008. With regard to iNOS expression, significant inhibition after 24 h of induction was only observed with extracts but not RA treatment, and the quercetin control was more active than the other treatments. At the same time, constitutive expression of COX-1 was not affected by any of the treatments.

DISCUSSION

Prunella vulgaris has been used as traditional and alternative therapy for minor acute inflammation and chronic inflammatory diseases for over two decades (1), but systematic scientific proof of its efficacy is limited. Whereas the majority of earlier research focused on the immune-regulatory, skin UV-damage protective, and antimicrobial effects of *P. vulgaris* water extracts (4, 23), our study showed that ethanol extracts from selected accessions of *P. vulgaris* significantly inhibited production of LPS-induced inflammatory mediators PGE2 and NO by RAW 264.7 mouse macrophages. This is in accordance with reports on anti-inflammatory activity of *P. vulgaris* ethanol extracts (3, 8, 24), whereas the water extract was attributed with putative immunostimulatory activity (4). However, to our knowledge, this is the first study to systematically demonstrate that *P. vulgaris* ethanol extracts from various accessions dose-dependently inhibited LPS-induced PGE2 and NO production without cytotoxicity. The water extracts from the same accessions had no effect on PGE2

production, but exerted mild inhibition on NO production. A past study of the water extracts used in the present research demonstrated the presence of abundant polysaccharides, associated with antiviral activity (5). To unveil whether polysaccharide was a common active component in both water and ethanol extracts, we tested the effect of aqueous re-extraction of the most active Ames 27664 ethanol extract against LPS-induced PGE2 and NO release and compared it to the original ethanol extract. This sequential extract did not significantly reduce PGE2 production, suggesting that polysaccharides did not play a role in the anti-inflammatory activity of the ethanol extract. We did not further study the water extracts due to their very limited activity in the study. Although most assayed ethanol extracts were able to attenuate both the LPS-induced production of PGE2 and NO, their potency on these two major inflammation mediators varied among accessions. This difference suggests that active compound(s) were produced unequally in these plant materials, even in the same accession from different harvests, and highlights the importance of chemical profile in anti-inflammatory activity. So far, accession Ames 27664 exerted the greatest inhibition on LPS-induced PGE2 and NO. In general, *P. vulgaris* ethanol extracts inhibited LPS-induced PGE2 and NO by 20–40% at concentrations as high as 20–30 $\mu\text{g}/\text{mL}$, which was a lower specific activity than we have seen in parallel studies of *Hypericum* (13) and *Echinacea* (14).

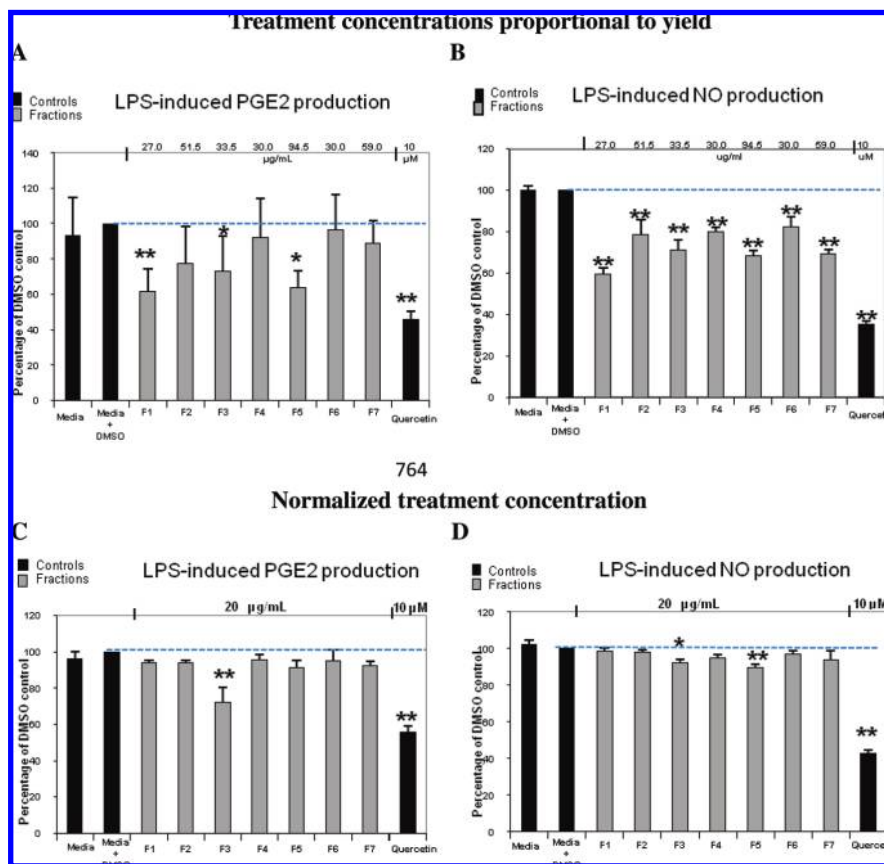


Figure 4. Seven fractions from ethanol extract of accession Ames 27664 (2007 harvest) were applied to RAW 264.7 macrophages at concentrations in proportion to their yield (**A, B**) or at a normalized concentration of 20 µg/mL (**C, D**) with or without 1 µg/mL LPS induction. Resulting PGE2 (**A, C**) and NO (**B, D**) productions are shown as percentage of media + DMSO vehicle control as mean ± SEM (N = 3). The 100% levels of PGE2 and NO were 3.89 ± 0.42 (**A**) and 4.32 ± 0.31 (**C**) ng/mL and 14.9 ± 0.84 (**B**) and 19.8 ± 1.19 (**D**) µM, respectively. Statistically significant differences are marked with asterisks (*, p < 0.05; **, p < 0.01). Quercetin was used as a positive control at 10 µM concentration. No difference was found in PGE2 and NO production without LPS stimulation among treatments, which is not shown.

Table 3. Abundance of Rosmarinic Acid in Ethanol Extracts from Different *P. vulgaris* Accessions^a

NCRPIS accession (Ames no.)	RA content in 30 µg/mL extracts (µM) (mean ± SEM)
27664 (2007 harvest)	2.7 ± 0.1*
27664	3.2 ± 0.2**
27665	2.0 ± 0.1
27748	3.7 ± 0.2**
28353	1.6 ± 0.1
28354	1.8 ± 0.2
28355	1.8 ± 0.1
28356	1.5 ± 0.1
28358	1.6 ± 0.2
28359	1.4 ± 0.1
28436	1.7 ± 0.2

^a HPLC was used to quantitate rosmarinic content in selected ethanol extracts from several *P. vulgaris* accessions. Accessions in which RA content is significantly higher than the overall average are labeled with asterisks (*, p < 0.05; **, p < 0.01; N = 9 or 10).

Production of PGE2 and NO by macrophages upon LPS induction was reported to be mediated by the toll-like receptor 4 (TLR-4) and subsequent nuclear factor-κB (NF-κB) downstream activation, which results in expression of COX-2 and iNOS (25). However, we saw somewhat different patterns between the effects of *P. vulgaris* extracts on PGE2 and NO production. For instance, Ames 27748 ethanol extract had no significant effect on PGE2 but imposed strong inhibition on NO

Table 4. Abundance of Rosmarinic Acid in Fractions from Ames 27664 *P. vulgaris* Ethanol Extract^a

fraction	RA content in the fractions (µM)	
	in fractions at concentrations proportional to yield	in 20 µg/mL fractions
1	0.5 ± 0.1	in 27.0 µg/mL 0.4 ± 0.1
2	1.3 ± 0.2	in 51.5 µg/mL 0.5 ± 0.1
3	0.9 ± 0.1	in 33.5 µg/mL 0.5 ± 0.2
4	0.8 ± 0.2	in 30.0 µg/mL 0.5 ± 0.1
5	5.2 ± 0.3**	in 94.5 µg/mL 1.1 ± 0.2**
6	0.8 ± 0.2	in 30.0 µg/mL 0.5 ± 0.1
7	1.6 ± 0.3*	in 59.0 µg/mL 0.5 ± 0.1

^a HPLC was used to quantitate rosmarinic content in all fractions from Ames 27664 *P. vulgaris* ethanol extract (2007 harvest). RA amount in fractions at concentrations in proportion to their yield and at 20 µg/mL are both shown. Fractions in which RA content is significantly higher than the overall average are labeled with asterisks (*, p < 0.05; **, p < 0.01; N = 9).

production. This could result from differential effects on transcriptional or/and post-transcriptional regulation of COX-2 and iNOS, which are worthy of future investigation.

Ethanol extract from Ames 27664 was fractionated into seven fractions with the intent of identifying possible active components. Three fractions demonstrated significant inhibition of LPS-induced PGE2 and NO production at concentrations in proportion to their yield from the extract. Among them, fractions 3 and 5, respectively, had significant effect on

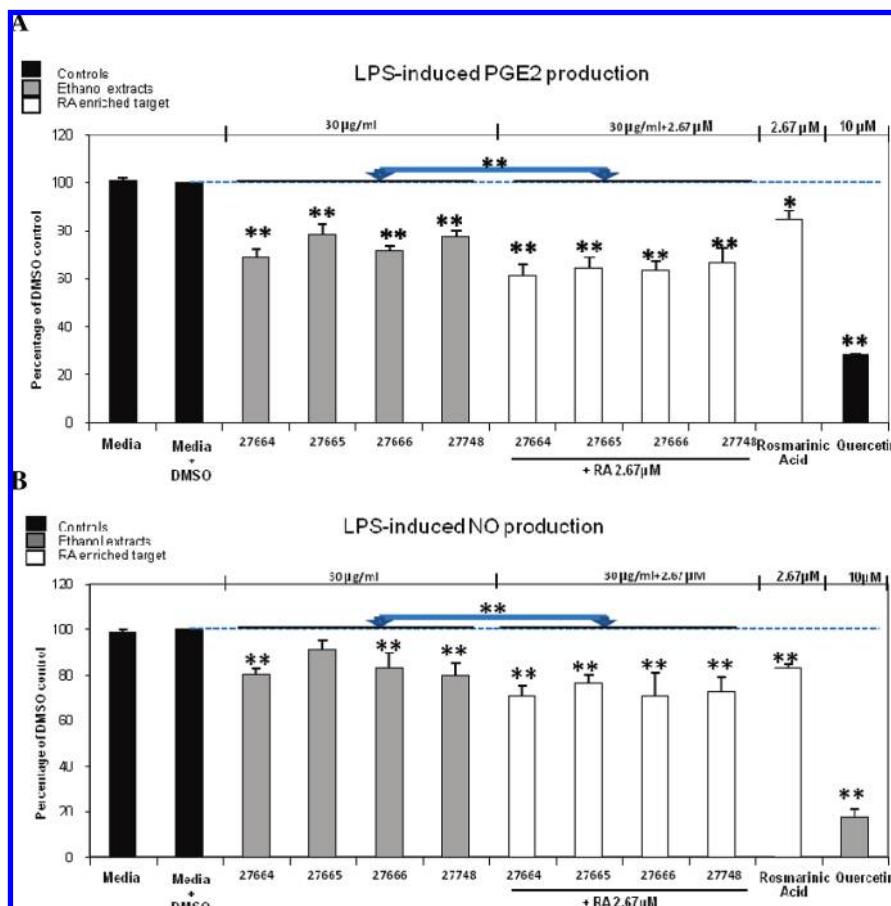


Figure 5. Ethanol extracts from accessions Ames 27664, 27665, 27666, and 27748 (2007 harvest) with and without additional 2.67 μM rosmarinic acid were applied to cells at 30 $\mu\text{g}/\text{mL}$ with and without 1 $\mu\text{g}/\text{mL}$ LPS induction. RA was also administered alone at the same concentration. Both PGE2 (A) and NO (B) productions are shown as percentage of media + DMSO vehicle control as mean \pm SEM ($N = 3$). The 100% levels of PGE2 and NO production were 2.92 ± 0.18 ng/mL and 14.4 ± 0.96 μM , respectively. Statistically significant differences compared to vehicle control are marked with asterisks (*, $p < 0.05$; **, $p < 0.01$). Comparison was also made between extracts and extracts + RA, as shown on top of the bars. Quercetin was used as a positive control at 10 μM concentration. No difference was found in PGE2 and NO production without LPS stimulation among treatments, which is not shown.

NO when treatment concentration was normalized to 20 $\mu\text{g}/\text{mL}$, whereas only fraction 3 significantly attenuated PGE2 level at this concentration. These observations revealed that active components of the ethanol extract were distributed in more than one fraction and act through different pathways. Considering that fraction 3 was the only fraction that significantly decreased both the PGE2 and NO productions at 20 $\mu\text{g}/\text{mL}$, it was expected to be relatively more abundant in active compounds or contain the major active constituent. On the other hand, fraction 5 could also be an important contributor to the observed extract anti-inflammatory activity, considering both its high yield from extract and its activity at higher concentration.

Rosmarinic acid, a polyphenol compound with known anti-inflammatory and antioxidant activities (26, 27), was of interest because of its presence in *P. vulgaris* ethanol extracts (16). Fraction 5 of the ethanol extract from Ames 27664 showed significant inhibition on both PGE2 and NO productions, whereas it contained most of the RA in the extract. This suggested RA being a probable anti-inflammatory component in *P. vulgaris* ethanol extracts. Pure RA treatment at the concentration that occurred in *P. vulgaris* ethanol extract significantly inhibited LPS-induced PGE2 and NO productions, although it did not explain all of the extract's activity as shown by the lower inhibitory potency. This confirmed RA as one active compound that partially accounted for the overall anti-inflammatory

effect of *P. vulgaris* ethanol extracts. This is in accordance with the recently published study on human gingival fibroblasts by Zdarilova et al., in which an aqueous ethanol extract of *P. vulgaris* and the corresponding RA component inhibited LPS-induced oxidative stress and expression of several pro-inflammatory enzymes including iNOS (8). Compared to their study, we had a lower abundance of RA content (1.73–3.88% of dry matter weight vs 9.0%) in the extract, which likely resulted from our using 95% ethanol for extraction instead of the 30% ethanol they used. According to Chizzola et al., an aqueous ethanol extract was more capable of concentrating flavonoids in *Thymus vulgaris* (28), which is likely the same case for *P. vulgaris* and may explain the lower RA in our extracts. The main reason that we used 95% ethanol was to minimize endotoxin extraction. Although our extract was not designed to enrich RA content, it was intriguing to see the RA effect at the extracted concentration. To see whether additional RA can further promote the extract activity, we supplemented the extracts with additional RA. As we expected, the RA-enriched extracts exhibited a stronger inhibition of the LPS-induced inflammatory response. We used two-way ANOVA with block to assess whether there was interaction between RA and other components in the extracts. No interaction was found ($p = 0.85$), suggesting the RA effect was additive instead of synergistic when it was added to the extracts. We also attempted to relate RA concentration to activity potencies of various accessions,

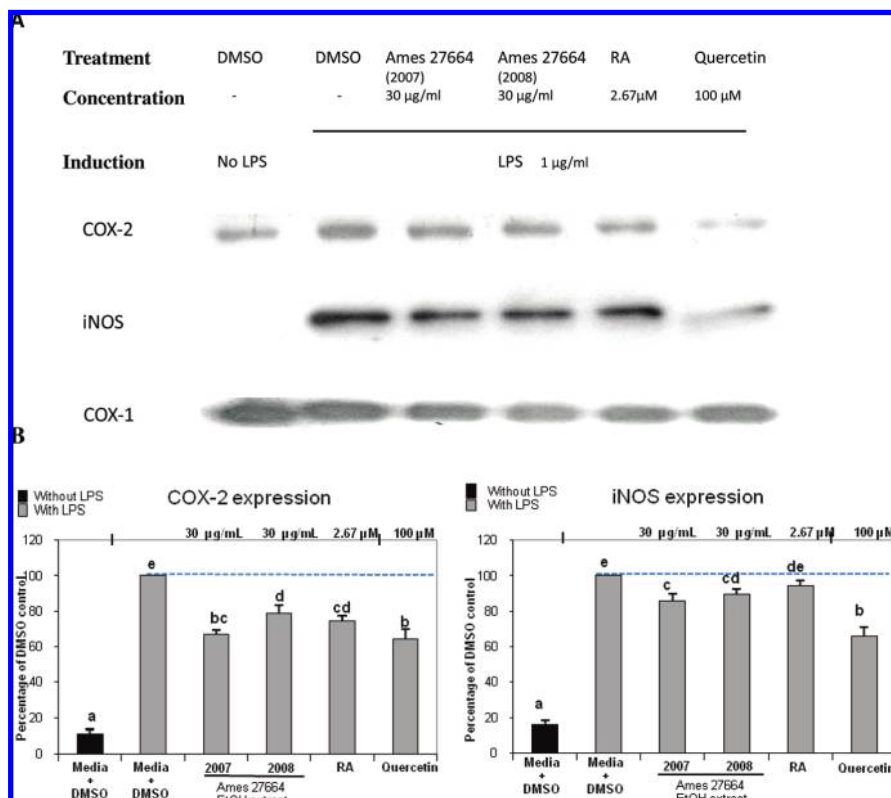


Figure 6. Treatments of controls and extracts or RA pure compound were applied to RAW 264.7 macrophages with or without LPS induction. Media + DMSO vehicle control was also used alone without induction. (A) shows a block of examples of COX-2 and iNOS protein expression level identified with Western blotting. Constitutively expressed COX-1 expression was used as reference. (B) demonstrates COX-2 and iNOS protein abundance quantified as arbitrary densities and showed as percentage of media + DMSO vehicle control in mean \pm SEM ($N = 4$). Values with statistically significant differences are denoted with different letters ($a < b < c < d < e$; $p < 0.05$). Quercetin positive control was used at 100 μM concentration.

but the resulting Pearson correlation coefficient was not significant for either PGE2 or NO inhibition ($r = 0.42$ and $p = 0.22$ for PGE2; $r = -0.26$ and $p = 0.4652$ for NO). This analysis, together with the observation that the Ames 27664 extract from the 2007 harvest had stronger activity but lower RA concentration compared to that from 2008 and the most active fraction 3 did not have the most RA, further predicted the existence of anti-inflammatory component(s) other than RA in these *P. vulgaris* ethanol extracts.

Ursolic acid is a known triterpene component in *P. vulgaris* plant material (18). UA concentration was 0.2 μM in 30 $\mu\text{g/mL}$ of the Ames 27664 ethanol extracts, far below the threshold for significant anti-inflammatory effect reported by Ryu et al. (10). This UA abundance was comparable to the results reported by Lee et al. that showed UA accounted for 0.05–0.2% of dry matter weight of ethanol extracts of 15 different *P. vulgaris* market samples (29). We did not detect UA in any of the fractions we studied. Despite the low abundance in the extracts, we examined the effect of pure UA compound on LPS-induced PGE2 and NO production by RAW 264.7 macrophages and did not see any effect at up to 1 μM concentration (data not shown). This ruled out UA as an independent active component of *P. vulgaris*. We have been using UA as positive control in cytotoxicity assay at 30 μM , but its cytotoxicity effect in the extracts we used is improbable due to the extremely low concentration.

COX-2 protein expression was significantly inhibited by *P. vulgaris* ethanol extracts and pure RA, whereas iNOS protein expression was only slightly attenuated by the extracts but not RA. At the same time, COX-1 protein expression was not

affected. This evidence suggests inhibition exists at the transcriptional level, possibly through NF- κ B signaling pathways. It is likely that there could be compounds other than RA in the extracts inhibiting the expression and/or the activity of the two critical enzymes in inflammation.

Although this study provides evidence for anti-inflammatory activity of *P. vulgaris* in this well-defined mouse macrophage model, additional research is required to demonstrate that these observations are relevant to human health and to demonstrate that *P. vulgaris* affects macrophages in vivo. For example, studies of bioavailability and long-term low-dose effects in vivo are needed, especially because only relatively high concentrations of *P. vulgaris* extract showed significant activity. For rosmarinic acid, bioavailability has been studied in a pig model by Jirovsky et al., who showed that after 91 days of feeding methanol extract of *P. vulgaris*, RA and RA metabolites (caffeic acid, etc.) were found in plasma at $>0.08 \mu\text{M}$ (30). Notably, this is considerably lower than the 2.67 μM dose we used. Therefore, our study indicated the potential anti-inflammatory activity of *P. vulgaris* but still awaits further investigation on its long-term in vivo impact.

In summary, ethanol extracts of *P. vulgaris* inhibited LPS-induced PGE2 and NO production in RAW 264.7 mouse macrophage cells. This activity was dose-dependent and varied among accessions and harvests. Rosmarinic acid, a polyphenol component of *P. vulgaris* ethanol extracts, showed independent anti-inflammatory activity when applied to cells at the same concentration as in the ethanol extract. However, RA could only partially explain the overall activity of the extract. Ethanol extracts from *P. vulgaris* attenuated both COX-2

and iNOS protein expression, whereas pure RA demonstrated inhibition on only COX-2. Further active component identification and bioavailability studies will help reveal more about *P. vulgaris* as an anti-inflammatory dietary supplement.

ABBREVIATIONS USED

RA, rosmarinic acid; UA, ursolic acid; PGE₂, prostaglandin E₂; NO, nitric oxide; TLR4, toll-like receptor 4; COX-2, cyclooxygenase-2; iNOS, nitric oxide synthase; NF- κ B, nuclear factor- κ B.

ACKNOWLEDGMENT

We give special thanks to members of the Center for Research on Botanical Dietary Supplements of Iowa State University for their continuous support. We also acknowledge the initial studies on *P. vulgaris* conducted by Dr. Mee-hye Kim of the Korean FDA. We also appreciate the analytical instrumentation support from Dr. Ann Perera, manager of the W. M. Keck Metabolomics Facility, at Iowa State University.

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Received for review July 9, 2009. Revised manuscript received October 19, 2009. Accepted October 23, 2009. This is a journal paper supported by Hatch Act and State of Iowa funds. This study was supported by Grant P50 AT004155-06 from the Office of Dietary Supplements, the National Center for Complementary and Alternative Medicine, National Institutes of Health. Mention of commercial brand names does not constitute an endorsement of any product by the U.S. Department of Agriculture or cooperating agencies.