**Effect of pecan phenolics on the release of nitric oxide from
murine RAW 264.7 macrophage cells**

Katherine S. Robbins,^a Phillip Greenspan,^b and Ronald B. Pegg^{a,*}

^a*Department of Food Science & Technology, College of Agricultural and Environmental
Sciences, The University of Georgia, 100 Cedar Street, Athens, GA 30602-2610, USA*

^b*Department of Pharmaceutical and Biomedical Sciences, College of Pharmacy, The University
of Georgia, Athens, GA 30602, USA*

Running Title: Effect of Pecan Phenolics on Inflammation Markers

*Corresponding author:
Dr. Ronald B. Pegg
Department of Food Science & Technology
The University of Georgia
100 Cedar Street
Athens, GA 30602-7610
USA
Tel: (706) 542-1099; Fax: (706) 542-1050; E-mail: rpegg@uga.edu

ABSTRACT

Inflammation is linked to numerous chronic disease states. Phenolic compounds have attracted attention because a number of these compounds possess anti-inflammatory properties. A phenolic crude extract was prepared from pecans and separated by Sephadex LH-20 column chromatography into low- and high-molecular-weight (LMW/HMW) fractions. Anti-inflammatory properties of these fractions were assessed in LPS-stimulated RAW 264.7 murine macrophage cells. NO and reactive oxygen species (ROS) production was monitored after 3 different experimental protocols: (1) pre-treatment with *E. coli* O111:B4 lipopolysaccharide (LPS); (2) pre-treatment with a pecan crude extract and its fractions; and (3) co-incubation of LPS with a pecan crude extract and its fractions. The LMW fraction displayed a dose-dependent decrease in NO production and a significant decrease from the LPS control in ROS production when cells were either co-incubated with or pre-treated with LPS. The phenolics were characterized by HPLC to help identify those responsible for the observed effect.

Keywords: Pecans; RAW 264.7; Inflammation; Griess assay; Proanthocyanidins (PACs); RP-HPLC; HPLC-ESI-MS/MS

1. Introduction

NF- κ B is a protein complex shown to play a key role in the inflammation process in macrophages and lymphocytes (Glass & Witztum, 2001). During normal physiological conditions, the protein complex is located in the cytosol of the cell as an inactive complex, I κ B-NF κ B (Baeuerle & Baltimore, 1996). The inflammatory process begins when one of several possible stimuli activates the cell. These stimuli can be exogenous (such as lipopolysaccharides, LPS) or endogenous (TFN- α or IFN- γ) (Terra et al., 2007; Rajapakse, Kim, Mendis, & Kim, 2008). The stimulation then causes the enzyme I κ B-kinase (I κ K) to phosphorylate the inactive complex. Once the complex is activated, it can pass into the nucleus where it can stimulate gene expression for the inducible nitric oxide synthase (*i*NOS) (Kretz-Remy, Mehlen, Mirault, & Arrigo, 1996; Aktan 2004; Rajapakse et al., 2008), and a marked increase in the production of cytokines and other inflammatory mediators (Barnes & Karin, 1997). Nitric oxide (NO), a somewhat-stable free radical, is thought to be a mediator of inflammation (Skoog et al., 2002) and is produced once the NF- κ B complex has been activated by extracellular stimuli (Terra et al., 2007; Kumar, Abbas, Fausto, & Aster, 2010). Moreover, it is generated during everyday activities to help mediate other biological functions in the human body (Skoog et al., 2002). The conditionally-indispensable amino acid L-arginine is converted to NO by one of three NO synthases: constitutive synthases, endothelial (*e*NOS) and neuronal nitric oxide synthases (*n*NOS), as well as inducible nitric oxide synthase (*i*NOS). Different from the constitutive synthases, *i*NOS is not found in uninjured cells (Mayer & Hemmens 1997; Alderton, Cooper, & Knowles, 2001). Upon any form of injury or inflammation, the NF- κ B complex will, however, initiate cytokines in the cell to begin producing *i*NOS (Qidwai & Jamal, 2010).

NO serves multiple purposes in the inflammation process (Laroux et al., 2001). It is a small molecule and is therefore capable of diffusing easily into cells. This enables the NO to travel from the site of formation to various sites of action (Kruidenier & Verspagel 2002; Aktan 2004). Because it is a free radical, NO has the potential to react with other free radicals found in cells. NO itself is not extremely reactive, but it does form a much more damaging reactive nitrogen species (RNS), peroxynitrite anion (ONOO^-) (Zhong, Chiou, Pan, & Shahidi, 2012). For this reason, the pro-oxidative NO has been shown to create oxidative damage (Epe, Ballmaier, Roussyn, Briviba, & Sies, 1996; Luperchio, Tamir, & Tannenbaum, 1996; Virgili, Kobuchi, & Packer, 1998). It is also believed that ROS may play a key role in the activation of NF- κ B (Brigelius-Flohé, Banning, Kny, & Böhl, 2004).

Studies have investigated the anti-inflammatory properties of pine bark extract (Virgili et al., 1998), cocoa procyanidins (Zhang et al., 2006) and a hydrophilic phenolic extract from pistachios (Gentile, Allegra, Angileri, Pintaudi, Livrea, & Tesoriere, 2012) in cell culture systems. Varying, and conflicting, results have been reported in regards to which extract constituents are most effective at reducing NO and ROS production. For instance, one study reports that HMW tannin constituents (Gentile et al., 2012) are the most effective, while others have demonstrated the effectiveness of monomers and dimers (Zhang et al., 2006; Park, Rimbach, Saliou, Valacchi, & Packer, 2000). Due to the moderately high **proanthocyanidin (PAC)** content of pecans, it was postulated that a phenolic crude extract from pecans would be excellent as inhibitors of the inflammatory process. The prepared pecan crude extract was further separated via Sephadex LH-20 column chromatography to give two phenolic fractions: a LMW and a HMW one. Through the combination of results from HPLC characterization and cell

culture assays, our objective is to indicate which compounds may be most effective as anti-inflammation agents.

2. Materials and methods

2.1. Chemicals and glassware

Sea sand, glass wool, dimethyl sulfoxide (DMSO), sodium carbonate, and ethanol (95%), as well as ACS-grade methanol, hexanes, and acetone plus HPLC-grade water, methanol and acetonitrile were acquired from Fisher Scientific Co., LLC (Suwanee, GA, USA). Glacial acetic acid was purchased from VWR International, LLC (Suwanee, GA, USA). *Escherichia coli* O111:B4 lipopolysaccharide (LPS), Triton X-100, fetal bovine serum (FBS), MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], Folin & Ciocalteu's phenol reagent and (+)-catechin hydrate were purchased from the Sigma-Aldrich Co. (St. Louis, MO, USA). Dulbecco's Modified Eagle Medium (DMEM/high glucose + sodium pyruvate), L-glutamine, penicillin-streptomycin, and phosphate-buffered saline (PBS) were ordered from Invitrogen, Inc. (Grand Island, NY).

2.2. Sample collection

Pecans (Desirable variety, random selection from five orchards in South Georgia) were harvested during the autumn of 2012 and shipped to the Department of Food Science & Technology, Athens, GA. The nuts were raw and remained in-shell through transport, packaging,

and storage. After arrival in Athens, the samples were placed in labelled pouches (Sealed Air Corporation, Elmwood Park, NJ), vacuum packed (Henkelman 600, Henkelman BV, The Netherlands) to help prevent oxidative degradation, and stored at -80 °C until analyzed. On the day of analysis, pecan samples were removed from the freezer, cracked, shelled, and tempered to room temperature to ensure a proper mass reading. Approximately 15 g of nutmeat were placed into a -20 °C freezer and allowed to partially refreeze to facilitate grinding. Each nut sample was then combined with ~65 g of washed sea sand and ground in a commercial coffee mill (Grind Central Coffee Grinder, Cuisinart, East Windsor, NJ), to a very fine powder using an intermittent pulsing technique. In this manner, oils were not expressed from the nutmeat during the particle size reduction process.

2.3. Extraction of phenolic compounds

A Soxhlet apparatus was employed to remove lipids from all pecan samples. The (CH₃)₂CO/H₂O/CH₃COOH solvent mixture (70:29.5:0.5 v/v/v, 100 ml) of Wu, Beecher, Holden, Haytowitz, Gebhardt, and Prior (2004) was used to extract phenolic compounds from the defatted nutmeat. Extraction was carried out according to Craft, Kosińska, Amarowicz, and Pegg (2010). Briefly, the contents in the flasks were heated at 50 °C for 30 min in an orbital-shaking water bath (New Brunswick Scientific, New Brunswick, NJ). The extraction was performed in triplicate, and supernatants were pooled. Acetone was evaporated from the supernatant using a Rotavapor. The aqueous portion was transferred to crystallization dishes, covered with filter paper, and placed in a -80 °C freezer until frozen. The samples were then lyophilized (Labconco Freezone 2.5 L freeze dryer, Labconco Corp., Kansas City, MS). The dried extract was placed in

amber-coloured vials, capped, and stored at 4 °C until ready for use. The entire extraction process was conducted in triplicate.

2.4. Fractionation of the phenolic extracts

Pecan phenolics were fractionated from the crude extracts according to Strumeyer and Malin (1975) with slight modifications. Briefly, a ~2 g sample of pooled pecan crude extracts from 5 independent preparations was mixed in a small volume of ~75% (v/v) ethanol and sonicated to ensure that it was completely dissolved. Using a Pasteur pipette, the sample was applied to the top of a chromatographic column packed with Sephadex LH-20 (bead size: 25-100 µm; Chromaflex column, 30 x 400 mm [I.D. x length], Kontes, Vineland, NJ, USA). LMW phenolic compounds were eluted with ~1.5 L of 95% (v/v) ethanol. Fractions (15 ml) were collected using a Beckman Coulter SC100 fraction collector (Fullerton, CA, USA). The eluent was then switched to 50% (v/v) aqueous acetone and a HMW phenolic fraction was eluted from the column with ~600 ml of this mobile phase.

2.5. Pooling of isolated fractions

Each 15 ml LMW phenolic fraction was analyzed using an Agilent 8453 UV/Vis DAD spectrophotometer (Agilent Technologies, Inc., Wilmington, DE) by scanning across a wavelength range of 200 - 400 nm. Spectral differences at key wavelengths (i.e., λ = 255 nm {ellagic acid and its derivatives}, 278 nm {phenolic acids, catechin, epicatechin}, 320 nm {phenolic acids of the *trans*-cinnamic acid family} and ~360 nm {flavonols}) were used as a

guide to group the collected fractions into 5 pooled lots (Fractions I-V); pooling was based on observed similarities and differences in the spectra of the samples. Further details of the pooling process as well as a depiction of the spectral characteristics for the resultant fractions are given by Robbins, Ma, Wells, Greenspan, and Pegg (2014). After pooling, ethanol was evaporated using a Rotavapor with the water bath set at 45 °C. To ensure complete removal of solvent and moisture, the pooled fractions were lyophilized with the Labconco freeze dryer and then stored in amber-glass bottles at 4 °C until analysis. Apropos the HMW fraction: acetone was also removed *in vacuo* and the aqueous residue lyophilized and stored as described above.

2.6. Total phenolics content (TPC) determination

The TPC assay of the ethanolic fractions was determined based on a method adapted from Swain and Hillis (1959), employing Folin & Ciocalteu's phenol reagent. Each fraction was diluted to 0.20 mg/ml in anhydrous methanol. The assay was performed using 1 ml of methanolic extract, 7.5 ml deionized water, 0.5 ml Folin & Ciocalteu's phenol reagent, and 1 ml of saturated sodium carbonate. The resultant solution was vortexed for 30 s. The tubes sat for 60 min to allow for maximal colour development. The absorbance of the resulting chromophore from this colorimetric reaction was measured at $\lambda = 750$ nm using the Agilent spectrophotometer. A standard curve was prepared using known concentrations of working solutions of (+)-catechin ranging from 1.6 to 8.0 µg/ml.

Based on the low TPCs of fractions I and II, fractions III to V were combined to give the LMW fraction used in the cell culture studies. Ethanolic solutions were prepared with phenolic levels of the following specifications: LMW – 2 mg (+)-catechin equivalents (CE)/ml in 70%

(v/v) ethanol; HMW– 5 mg CE/ml in 50% (v/v) ethanol; and crude extract – 5 mg/ml CE in 75% (v/v) ethanol. Differences in the ethanol concentrations were necessary to ensure complete solubilization.

2.7. RP-HPLC and hydrophilic interaction liquid chromatography (HILIC) analyses

RP-HPLC assays were carried out with a reversed-phase Luna C18(2) column (4.6×250 mm, 5- μ m particle size; Phenomenex, Torrance, CA, USA) and a gradient elution consisting of mobile phase A ($\text{H}_2\text{O}/\text{CH}_3\text{CN}/\text{CH}_3\text{COOH}$, 93:5:2, v/v/v) and mobile phase B ($\text{H}_2\text{O}/\text{CH}_3\text{CN}/\text{CH}_3\text{COOH}$, 58:40:2, v/v/v) from 0 to 100% B over a 50 min period at a flow rate of 1 ml/min. Detection wavelengths were $\lambda = 255$ nm (ellagic acid and ellagic acid derivatives), 280 nm (phenolic acids, (+)-catechin, (–)-epicatechin), 320 nm (phenolic acids, notably of the *trans*-cinnamic acid family), and 360 nm (flavonols). The proanthocyanidins were separated based on their degree of polymerization by HILIC with a PrincetonSPHER DIOL column (250 mm $\times$ 4.6 mm, 5 μ m particle size, 60 Å; Princeton Chromatography, Inc., Cranbury, NJ, USA), a fluorescence detector ($\lambda_{\text{ex/em}} = 276/316$ nm), and a gradient elution consisting of mobile phase (A) $\text{CH}_3\text{CN}/\text{CH}_3\text{COOH}$ (98:2, v/v) and (B) $\text{CH}_3\text{OH}/\text{H}_2\text{O}/\text{CH}_3\text{COOH}$ (95:3:2, v/v/v). At 1.0 ml/min the gradient was 0–35 min, 0–40% B; held for 5 min; 40–45 min, 40–0% B; and then an additional 5 min hold in order to equilibrate the system. Further details on the analytical conditions as well as the further HPLC-ESI-MS/MS analyses are given by Robbins et al. (2014).

2.8. Cell culture model

Murine RAW 264.7 macrophage cell lines were purchased from the American Cell Culture Collection (Manassas, VA). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % endotoxin-free, heat-inactivated fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 µg/ml), and L-glutamine (2 mM) and maintained at 37 °C in a humidified 5% (v/v) CO₂ incubator. After scraping cells from a Corning 25-cm² culture flask, cells were plated (3.0×10^5) in MULTIWELL™ 24-well flat bottom tissue culture-treated dishes (Falcon, Franklin Lakes, NJ) and incubated until confluent. Three different treatment application methods were employed: (i) the co-incubation treatment involved adding 0.5 ml of 100 ng/ml LPS in DMEM media supplemented with 5 % FBS. The cells were incubated at 37 °C for 1 h. Then, 0.5 ml of the three ethanolic phenolic solutions (i.e., for the crude extract and its LMW and HMW fractions) with phenolic concentrations of either 25, 50, or 100 µg CE/ml (prepared in 5% FBS DMEM) were added to the appropriate well in quadruplicate, and the cells were incubated for 18 h; (ii) the extract pre-treatment involved adding 0.5 ml of each of the three ethanolic phenolic solutions prepared in 5% FBS DMEM and incubating at 37 °C for 1 h. The cells were then washed twice with PBS and 1 ml of 100 ng/ml LPS in DMEM with 5% FBS was added, and the cells were incubated for an additional 18 h; and (iii) the final treatment, a pre-treatment with LPS, involved adding the 100 ng/ml LPS in DMEM with 5% FBS and incubating for 1 h. At this point, the cells were washed twice and then the three ethanolic phenolic solutions in DMEM were added for the final 18 h. For (i), (ii) and (iii), ethanol concentrations were held constant across all treatments and concentrations. Final concentrations of the extracts in the wells were 12.5, 25, and 50 µg CE/ml.

2.9. MTT cell viability assay

Cell viability was determined using the method of Gentile et al. (2012), with slight modifications. Briefly, the cells were washed with PBS (pH 7.4) following the 18 h incubations at 37 °C. Then, 300 µL of FBS-free DMEM medium containing 5 mg/ml MTT was added to each well, and the plate was incubated for 4 h at 37 °C. The medium was discarded after incubation and the cells were washed again with PBS. DMSO (1 ml) was added to each well and the plate was allowed to sit until all of the formazan blue formed in the cells was dissolved. The absorbance at $\lambda = 540$ nm of each well was measured in a spectrophotometer (Beckman DU 650 Spectrophotometer, Beckman Coulter, Inc., Indianapolis, IN).

2.10. Measurement of reactive oxygen species (ROS)

Production of ROS was measured using a method described by Zhang, Bull, Fisher, Cotruvo, and Cummings (2011) with slight modifications. Briefly, 300 µl of 10 µM CM-H₂DCFDA in 1% (v/v) ethanol (final concentrations) were added to each well 30 min prior to harvesting. The plate was incubated for an additional 60 min, after which the medium was removed and cells were detached using 300 µL of 0.5% Triton X-100. An aliquot (100 µL) was removed from each well and placed in a 96-well microtiter plate. The fluorescence was measured using a BMG FLUOstar Omega (Ω) microplate reader (BMG Laboratories, Cary, NC) at an excitation wavelength of 485 nm and an emission wavelength of 520 nm.

2.11. Cellular release of nitric oxide (NO)

After 18 h of incubation at 37 °C, the culture media was pipetted off, centrifuged at 1500 × g for 10 min with an Eppendorf® 5415D microcentrifuge (Marshall Scientific, Hampton, NH, USA), and used to measure the NO generated by the cells from LPS activation of the NF-κB pathway. NO production was measured as nitrite (NO₂⁻) using a Molecular Probes Griess Reagent Kit (Life Technologies, Eugene, OR). The Griess reagent was prepared by combining equal parts (v/v) of *N*-(1-naphthyl)ethylenediamine•dihydrochloride and sulfanilic acid. To a microplate, 20 µL of the Griess reagent, 150 µL of sample or standard, and 130 µL of deionized water were added to each well. Sodium nitrite was the standard with ten working concentrations ranging from 1 to 100 µM. The plate was allowed to rest at room temperature for 30 min to allow for maximal colour development of the azo dye. The absorbance of each well was read at λ = 548 nm using the spectrophotometric setting of the microplate reader, and the data was converted to nitrite concentrations based on the standard curve.

2.12. Statistical analyses

Differences in the means of each ethanolic extract were determined for each of the measured quantities with the PROC GLM procedure. Differences in means from control were determined with the means procedure and a Tukey's mean separation test with *P* < 0.05 using the Statistical Analysis System software, Version 9.2 (SAS Institute, Cary, NC).

3. Results

272 Triplicate preparations of three extracts (LMW fraction {combined fractions III to V}, HMW
273 fraction, and the phenolic crude extract) were tested for their capacity to reduce cellular ROS
274 generation and the production of a cellular inflammatory biomarker (NO). The production of NO
275 was analyzed under three different experimental conditions: (i) co-incubation of LPS and the
276 pecan extracts for 18 h; (ii) pre-treatment of the cells with LPS followed by a washing step and
277 application of the extracts; and (iii) pre-treatment with the extracts followed by a washing step
278 and then incubation with LPS. To ensure that proper activation of the cells with the LPS
279 occurred, a control sample was included in every experiment. As expected, the addition of LPS
280 caused a dramatic increase in NO and ROS production in the cells. The level of ethanol in the
281 cells was controlled at a final concentration of 0.9% in order to remove ethanol concentration as
282 a variable in the experiment. No cell death was observed at this low ethanol concentration.

283 The level of NO production was measured as nitrite production using the Griess assay. For
284 the co-incubation experiments, the LMW extracts showed a classical dose-dependent decrease in
285 NO production with declines of 22, 29, and 78% with the 12.5, 25, and 50 $\mu\text{g CE/ml}$ extracts,
286 respectively (Figure 1A). The 25 and 50 $\mu\text{g CE/ml}$ tests were shown to be significantly different
287 from the control ($P < 0.05$). The HMW and crude extracts did not display significant reductions
288 compared to the control ($P > 0.05$) for any of the concentrations investigated. For the
289 experiments with the pre-treatment of the extracts, there were no significant differences from
290 control for any of the concentrations or extracts investigated (Figure 1B). Moreover, there was
291 no dose-dependent effect observed for any of the extracts. In the pre-treatment with LPS
292 experiments, the results were similar to the co-incubation experiments. A dose-dependent
293 decrease of NO production in the presence of the LMW extracts was once again observed
294 (Figure 1C). All three concentrations of the LMW extract, 12.5, 25, and 50 $\mu\text{g CE/ml}$, displayed

significant decreases from the LPS control ($P < 0.05$). The decreases were 28, 37, and 77 % for the 12.5, 25, and 50 μg CE/ml extracts, respectively. The lowest concentration for both the HMW and crude extracts also reduced NO production, though these effects were small (11 and 14%, respectively). It should be noted that there was no dose dependent effect observed with either of these extracts. To test the cytotoxicity of the extracts, all treatments were subjected to the MTT viability assay. The 12.5, 25, and 50 μg CE/ml exhibited decreases from LPS control of 9.5, 12.0, and 14.7 %, respectively (Figure 2). It should be noted that only the 25, and 50 μg CE/ml were significant compared to the LPS control.

The cellular production of ROS was evaluated using $\text{H}_2\text{-DCFDA}$, a non-fluorescent compound that fluoresces upon reaction with a ROS. A dramatic difference in fluorescence was observed between control cells and cells treated with LPS. The middle concentration, 25 μg CE/ml of each of the three extracts, was tested in cells pre-treated with LPS (Figure 3). While no significant differences ($P > 0.05$) were observed for the HMW and crude extracts, a significant decrease was observed for the LMW extracts ($P < 0.05$). These results are in agreement with those results observed in the NO production experiments (Figure 1). The ROS production was decreased by almost 50% with incubation of the LMW extract. The ROS decreased from 21.2×10^4 fluorescence intensity for the LPS control samples to 11.4×10^4 fluorescence intensity for the LMW samples.

To determine the phenolic compounds which may be present in these LMW fractions, the fractions present in the extract were analyzed using RP-HPLC and the major constituents were identified and quantified, as presented by Robbins et al. (2014). The results have been summarized in Table 1. Two main groups of phenolic acids were identified across the combined three fractions utilized for the cell culture investigation: (+)-catechin and ellagic acid (including

their derivatives). An example chromatogram at both 255 and 280 nm of the phenolic compounds isolated from a fraction III sample (i.e., part of the LMW sample) is highlighted in Figure 4. The phenolics were separated and identified by HPLC-ESI-MS/MS as reported by Robbins et al. (2014).

These compounds were quantified in each sample and reported as mg phenolic/g fraction. All of the phenolic acid derivatives, such as an ellagic acid pentose, were quantified as equivalents and summed to produce a total for that phenolic acid. It was determined that fraction III contained 35.5 mg catechin/g fraction and 41.3 mg ellagic acid/g fraction, while fraction IV possessed 4.97 mg ellagic acid/g fraction. Fraction V did not appear to contain any phenolic acids or monomers identifiable by RP-HPLC. HILIC was therefore employed to further investigate fraction V. Monomers and dimers were identified (chromatogram not shown), but no compounds with higher degrees of polymerization were detected.

4. Discussion

The moderately high levels of beneficial lipids (*i.e.* MUFAs, PUFAs, and tocopherols) have prompted investigation into their effect on inflammation (Gopinath, Buyken, Flood, Empson, Rohtchina, & Mitchell, 2011). Recent investigations concerning the phenolic constituents of commodities such as pine extract (Virgili et al., 1998), pistachios (Gentile et al., 2012), and tea extracts (Zhong et al., 2012) have shown that their PACs and other phenolic compounds can act as anti-inflammatory mediators.

A randomized control clinical trial reported by Hudthagosol, Haddad, McCarthy, Wang, Oda, and Sabaté (2011) demonstrated that phenolic constituents of pecans are absorbable and impart

antioxidant activity *in vivo*, as assessed by significant increases in ORAC_{FL} and FRAP values of human plasma and reductions in oxidized LDL postprandially over an 8 h period. Unfortunately, no information is available on the potential anti-inflammatory properties of pecans. The present study is the first to investigate the use of pecan phenolic extracts as mediators of inflammation in RAW 264.7 macrophage cells. Throughout our investigation, it was determined that the LMW fraction of pecan crude extract displays a dose-dependent decrease in the NO production in LPS-stimulated RAW 264.7 macrophage cells. This dose-dependent response was observed for both the co-incubation of the extract with LPS and pre-treatment of LPS prior to the addition of the extract (Figure 1). In fact, similar results were noted in both cases. No significant differences ($P > 0.05$) from the LPS control were observed for both the HMW fraction and the crude extract in any of the NO trials, except for the most dilute concentration when LPS was pre-incubated with the cells. The LMW fraction also exhibited a significant reduction ($P < 0.05$) in ROS production from that of the LPS control (Figure 3). No significant differences were observed from the LPS control for either the HMW or the crude extract samples.

Other studies investigating the anti-inflammatory properties of natural products have produced contradictory results in terms of the compounds most likely responsible for the observed anti-inflammatory effect. Our results support those articles that show this effect is most likely due to phenolic acids and small condensed tannin/PAC compounds (Zhang et al., 2006; Park et al., 2000); whereas, other studies point to the high-molecular-weight tannin fractions as being responsible for decreasing inflammation (Gentile et al., 2012). The only similar study with tree nuts examined the effect of a pistachio phenolic extract on the LPS-induced inflammatory response of RAW 264.7 macrophage cells (Gentile et al., 2012). Gentile et al. (2012) reported

that the polymeric PACs displayed a much stronger effect than that of the smaller oligomeric PACs.

On the other hand, Park et al. (2000) conducted an experiment using commercial standards of compounds with varying degrees of polymerization (*i.e.*, catechin and epicatechin monomers, dimers, and trimers). These authors found that the monomers were the most effective at reducing the level of NO production in cells stimulated by IFN- γ , a similar inflammatory stimulator to LPS. It was also reported that when a commercial trimer (PCA C2) was used in the cell culture experiment, there actually was an increase in the production of NO! These results are similar to those observed in this study. It should be noted that the LMW fraction of pecans contained a high proportion of catechin monomers (particularly Fraction IV and V). An additional study conducted by Zhang et al. (2006) showed that COX-2, an additional enzyme linked to the inflammatory process, expression levels were greatly reduced by procyanidin B2 in human monocyte cells. Based upon the results of the HILIC characterization, fraction V did contain some monomeric and dimeric compounds, but no trimeric or larger compounds. It is suspected that the ethanol may have eluted some of the lower-molecular-weight tannin compounds. The effect of the LMW fraction of pecans can be attributed to not only the presence of ellagic acid and catechin compounds (Figure 4 and Table 1), but also to the inclusion of some of these LMW tannin constituents.

As previously mentioned, one argument for the effect of phenolics on curbing NO and ROS production is cytotoxicity. Using the MTT cytotoxicity study, the LMW extract showed small, significant differences ($P < 0.05$) from the LPS control in causing cytotoxicity but only for the two highest concentrations of 25 and 50 $\mu\text{g CE/ml}$. There were no significant differences observed between the three treatment concentrations; the authors do not believe that this is the

explanation for the LMW fraction-lowering of NO production. Another possible explanation is an interaction between the phenolic compounds and LPS. It has been suggested that the phenolic compounds might bind to the LPS making it difficult for the LPS to successfully initiate inflammation in the cells (Delehanty, Johnson, Hickey, Pons, & Ligler, 2007; Terra et al., 2007; Park et al., 2000). For this reason, the pre-treatment LPS experiments were performed, which are not utilized in many such studies. In this study, there were no large differences observed between the pre-treatment with LPS and the co-incubation of LPS and extracts experiments.

The importance of the phenolic extract concentration on the effect of NO and ROS production has been documented (Gentile et al., 2012). In the present study, the dose-dependent observations from the LMW extracts prove that concentration did, indeed, play a key role in the efficacy of the extract. In addition, when the cells were pretreated with different concentrations of extracts for 1 h, washed and then treated with LPS, no significant differences ($P > 0.05$) were observed in any of the assays. This stands in contrast to the inhibition observed in the production of inflammatory mediators when hydrophilic phenolic extracts from pistachios were pre-incubated with macrophages for only 1 h (Gentile et al., 2012).

Conclusion

In summary, the present study focused on the effect of different phenolic compounds isolated from pecans as inflammatory mediators. Phenolic acids and small condensed tannins found in the LMW fraction of pecans, rather than the HMW tannin constituents, were shown to have a dose-dependent effect at curbing inflammation in LPS-stimulated RAW 264.7 cells. Future studies are necessary to determine the mechanisms for the observed effects. Possible mechanisms proposed in the literature include changes in the expression of both COX-2 and iNOS enzymes, changes in the activation of NF- κ B, and changes in the level of mRNA expression (Virgili et al., 1998;

Zhang et al., 2006; Pan et al., 2008; Park et al., 2010; 2012). The antioxidant activity of the phenolic compounds in pecans may act in quenching key ROS which are involved in the activation of the NF- κ B complex.

Acknowledgments

The authors would like to acknowledge the Georgia Agricultural Commodity Commission for Pecans (GACCP) and the USDA-NIFA-SCRI Award No. 2011-51181-30674 for funding this research. Use of the Proteomics and Mass Spectrometry (PAMS) core facility under the direction of Dr. Dennis Phillips of UGA's Department of Chemistry is greatly appreciated; in particular, thanks are extended to Dr. Kevin D. Clark and Mr. Yi Gong for assistance with the LC-MS/MS analyses.

References

- Aktan, F. (2004). iNOS-mediated nitric oxide production and its regulation. *Life Sciences*, 75, 639–653.
- Alderton, W. K., Cooper, C. E., & Knowles, R. G. (2001). Nitric oxide synthases: structure, function and inhibition. *Biochemical Journal*, 357, 593–615.
- Barnes, P. J. & Karin, M. (1997). Nuclear factor- κ B – A pivotal transcription factor in chronic inflammatory diseases. *The New England Journal of Medicine*, 336, 1066–1071.
- Baeuerle, P. A. & Baltimore, D. (1996). NF- κ B: Ten years after. *Cell*, 87, 13–20.

431 Brigelius-Flohé, R., Banning, A., Kny, M., & Böhl, G.-F. (2004). Redox events in interleukin-1
 432 signaling. *Archives of Biochemistry and Biophysics*, 423, 66–73.

433 Craft, B. D., Kosińska, A., Amarowicz, R., & Pegg, R. B. (2010). Antioxidant properties of
 434 extracts obtained from raw, dry-roasted, and oil-roasted US peanuts of commercial
 435 importance. *Plant Foods **for** Human Nutrition*, 65, 311–318.

436 Delehanty, J. B., Johnson, B. J., Hickey, T. E., Pons, T., & Ligler, F. S. (2007). Binding and
 437 neutralization of lipopolysaccharides by plant proanthocyanidins. *Journal of Natural*
 438 *Products*, 70, 1718–1724.

439 Epe, B., Ballmaier, D., Roussyn, I., Briviba, K., & Sies, H. (1996). DNA damage by
 440 peroxynitrite characterized with DNA repair enzymes. *Nucleic Acids Research*, 24, 4105–
 441 4110.

442 Glass, C. K. & Witztum, J. L. (2001). Atherosclerosis: The road ahead. *Cell*, 104, 503–516.

443 Gentile, C., Allegra, M., Angileri, F., Pintaudi, A. M., Livrea, M. A., & Tesoriere, L. (2012).
 444 Polymeric proanthocyanidins from Sicilian pistachio (*Pistacia vera* L.) nut extract inhibit
 445 lipopolysaccharide-induced inflammatory response in RAW 264.7 cells. *European Journal*
 446 *of Nutrition*, 51, 353–363.

447 Gopinath, B., Buyken, A. E., Flood, V. M., Empson, M., Roachchina, E., & Mitchell, P. (2011).
 448 Consumption of polyunsaturated fatty acids, fish, and nuts and risk of inflammatory disease
 449 mortality. *The American Journal of Clinical Nutrition*, 93, 1073–1079.

450 Hudthagosol, C., Haddad, E. H., McCarthy, K., Wang, P., Oda, K., & Sabaté, J. (2011). Pecans
 451 acutely increase plasma postprandial antioxidant capacity and catechins and decrease LDL
 452 oxidation in humans. *The Journal of Nutrition*, 141, 56–62.

453 Kretz-Remy, C., Mehlen, P., Mirault, M.-E., & Arrigo, A.-P. (1996). Inhibition of I κ B- α
454 phosphorylation and degradation and subsequent NF- κ B activation by glutathione peroxidase
455 overexpression. *The Journal of Cell Biology*, 133, 1083–1093.

456 Kruidenier, L. & Verspaget, H. W. (2002). Review article: oxidative stress as a pathogenic factor
457 in inflammatory bowel disease – radicals or ridiculous? *Alimentary Pharmacology &*
458 *Therapeutics*, 16, 1997–2015.

459 Kumar, V., Abbas, A. K., Fausto, N., & Aster, J. (2010). Robbins and Cotran Pathologic Basis of
460 Disease, Eighth Edition; Saunders Elsevier: Philadelphia, PA.

461 Laroux, F. S., Pavlick, K. P., Hines, I. N., Kawachi, S., Harada, H., Bharwani, S., et al. (2001).
462 Role of nitric oxide in inflammation. *Acta Physiologica Scandinavica*, 173, 113–118.

463 Luperchio, S., Tamir, S., & Tannenbaum, S. R. (1996). NO-induced oxidative stress and
464 glutathione metabolism in rodent and human cells. *Free Radical Biology & Medicine*, 21,
465 513–519.

466 Mayer, B. & Hemmens, B. (1997). Biosynthesis and action of nitric oxide in mammalian cells.
467 *Trends in Biochemical Sciences*, 22, 477–481.

468 Pan, M.-H., Chang, Y.-H., Tsai, M.-L., Lai, C.-S., Ho, S.-Y., Badmaev, V., et al. (2008).
469 Pterostilbene suppressed lipopolysaccharide-induced up-expression of iNOS and COX-2 in
470 murine macrophages. *Journal of Agricultural and Food Chemistry*, 56, 7502–7509.

471 Park, Y. C., Rimbach, G., Saliou, C., Valacchi, G., & Packer, L. (2000). Activity of monomeric,
472 dimeric, and trimeric flavonoids on NO production, TNF- α secretion, and NF- κ B-dependent
473 gene expression in RAW 264.7 macrophages. *FEBS Letters*, 465, 93–97.

474 Park, H.-J., Jeon, B. T., Kim, H. C., Roh, G. S., Shin, J.-H., Sung, N.-J., et al. (2012). Aged red
475 garlic extract reduces lipopolysaccharide-induced nitric oxide production in RAW 264.7

476 macrophages and acute pulmonary inflammation through haeme oxygenase-I induction. *Acta*
477 *Physiological*, 205, 61–70.

478 Qidwai, T. & Jamal, F. (2010). Inducible nitric oxide synthase (*iNOS*) gene polymorphism and
479 disease prevalence. *Scandinavian Journal of Immunology*, 72, 375–387.

480 Rajapakse, N., Kim, M.-M., Mendis, E., & Kim, S.-K. (2008). Inhibition of inducible nitric oxide
481 synthase and cyclooxygenase-2 in lipopolysaccharide-stimulated RAW264.7 cells by
482 carboxybutyrylated glucosamine takes place via down-regulation of mitogen-activated
483 protein kinase-mediated nuclear factor- κ B signaling. *Immunology*, 123, 348–357.

484 Robbins, K. S., Ma, Y., Wells, M. L., Greenspan, P., & Pegg, R. B. (2014). Separation and
485 characterization of phenolic compounds from U.S. pecans by liquid chromatography–tandem
486 mass spectrometry. *Journal of Agricultural and Food Chemistry*, 62, 4332–4341.

487 Skoog, T., Dichtl, W., Boquist, S., Skoglund-Andersson, C., Karpe, F., Tang, R., et al. (2002).
488 Plasma tumour necrosis factor- α and early carotid atherosclerosis in healthy middle-aged
489 men. *European Heart Journal*, 23, 376–383.

490 Strumeyer, D. H. & Malin, M. J. (1975). Condensed tannins in grain sorghum: Isolation,
491 fractionation, and characterization. *Journal of Agricultural and Food Chemistry*, 23, 909–
492 914.

493 Swain, T. & Hillis, W. E. (1959). The phenolic constituents of *Prunus domestica*. I.–The
494 quantitative analysis of phenolic constituents. *Journal of the Science of Food and*
495 *Agriculture*, 10, 63–68.

496 Terra, X., Valls, J., Vitrac, X., Mérrillon, J.-M., Arola, L., Ardèvol, A., et al. (2007). Grape-seed
497 procyanidins act as antiinflammatory agents in endotoxin-stimulated RAW 264.7

498 macrophages by inhibiting NFκB signaling pathway. *Journal of Agricultural and Food*
 499 *Chemistry*, 55, 4357–4365.

500 Virgili, F., Kobuchi, H., & Packer, L. (1998). Procyanidins extracted from *Pinus maritima*
 501 (Pycnogenol®): Scavengers of free radical species and modulators of nitrogen monoxide
 502 metabolism in activated murine RAW 264.7 macrophages. *Free Radical Biology &*
 503 *Medicine*, 24, 1120–1129.

504 Wu, X. L., Beecher, G. R., Holden, J. M., Haytowitz, D. B., Gebhardt, S. E., & Prior, R. L.
 505 (2004). Lipophilic and hydrophilic antioxidant capacities of common foods in the United
 506 States. *Journal of Agricultural and Food Chemistry*, 52, 4026–4037.

507 Zhang, W.-Y., Liu, H.-Q., Xie, K.-Q., Yin, L.-L., Li, Y., Kwik-Urbe, C. L., et al. (2006).
 508 Procyanidin dimer B2 [epicatechin-(4β-8)-epicatechin] suppresses the expression of
 509 cyclooxygenase-2 in endotoxin-treated monocytic cells. *Biochemical and Biophysical*
 510 *Research Communications*, 345, 508–515.

511 Zhang, X., Bull, R. J., Fisher, J., Cotruvo, J. A., & Cummings, B. S. (2011). The synergistic
 512 effect of sodium chlorite and bromochloroacetic acid on BrO₃⁻-induced renal cell death.
 513 *Toxicology*, 289, 151–159.

514 Zhong, Y., Chiou, Y.-S., Pan, M.-H., & Shahidi, F. (2012). Anti-inflammatory activity of
 515 lipophilic epigallocatechingallate (EGCG) derivatives in LPS-stimulated murine
 516 macrophages. *Food Chemistry*, 134, 742–748.

Table 1. Phenolics content of LMW and HMW fractions from a pecan crude acetonc extract isolated by Sephadex LH-20 column chromatography.

Fraction ^a	TPC ^b (mg (+)-catechin equivalents/g fraction)	Catechin ^c	Catechin derivatives identified ^d	Ellagic Acid ^e	Ellagic acid derivatives identified	Total PACs ^f	PACs identified ^g
LMW	182 ± 1	35.5	(epi)gallocatechin, (+)-catechin	46.3	Ellagic acid pentose, digalloyl ellagic acid, ellagic acid, valoneic acid dilactone hydrate, methyl ellagic acid hexoside, ellagic acid galloyl pentose, ellagic acid HHDP glucose	31.1 ^h	PD2A PD2B
HMW	600 ± 9	N/A	N/A	N/A	N/A	83.5	DP of 2-6 Consisting of: all (epi)catechin or a combination of (epi)catechin and (epi)gallocatechin

^a LMW fraction was eluted from the Sehpandex LH-20 column with 95% (v/v) ethanol, whereas the HMW fraction was eluted using 50% (v/v) aqueous acetone.

^bResults are reported as mean ± standard deviation based on triplicate measurements.

^cCatechin values include free (+)-catechin, catechin hexoside, and (*epi*)catechin gallate. The data is expressed as mg catechin equivalents (CE)/g fraction.

^dCatechin and ellagic acid derivatives identified by RP-LC-MS/MS as described by Robbins *et al.* (2014).

^eEllagic acid contents include free ellagic acid and all other ellagic acid derivatives. All data is reported as mg ellagic acid equivalents/g fraction.

^fCharacterized by hydrophilic interaction liquid chromatography (HILIC) and quantification completed using commercial standards with varying degrees of polymerization. All quantities are expressed as mg compound/g tannin fraction.

^gPAC identification as described by Robbins *et al.* (2014).

^hN/A means not applicable. This data was not collected in our analyses due to restrictions of sample composition.

FIGURE CAPTIONS

- Figure 1. The effect of pecan extracts on NO production in murine RAW 264.7 macrophage cells: (A) co-incubated with LPS; (B) pretreated with the extracts; and (C) pretreated with LPS. The symbols are as follows: LPS control, ; low-molecular-weight (LMW) fraction, ; high-molecular-weight tannin fraction, ; and crude extract, . Mean values are reported with bars indicating standard error. Asterisks (*) indicate significant differences from the LPS control ($P < 0.05$), as determined by Tukey's mean separation.
- Figure 2. The effect of LMW pecan extracts on cellular viability. The symbols are as follows: LPS control, ; and low-molecular-weight (LMW) fraction, . Mean absorbance values reported with bars indicating standard error. Asterisks (*) indicate significant differences from the LPS control ($P < 0.05$), as determined by Tukey's mean separation test.
- Figure 3. The effect of pecan extracts (25 μg CE/mL) on mean ROS production. The symbols are as follows: LPS control, ; low-molecular-weight (LMW) fraction, ; high-molecular-weight tannin fraction, ; and crude extract, . Mean values are reported with bars indicating standard error. Asterisks (*) indicate significant differences from the LPS control ($P < 0.05$), as determined by Tukey's mean separation test.

Figure 4. Reversed-phase HPLC chromatogram at $\lambda = 255$ (dashed lines, in red for the PDF) and 280 nm (solid black lines) of fraction III isolated from a defatted pecan crude acetonic extract on a Sephadex LH-20 column using 95% (v/v) ethanol. The phenolic acids identified were as follows: (1) gallic acid; (2) an ellagic acid pentose; (3) (epi)catechin gallate; (4) ellagic acid; (5) valoneic acid dilactone hydrate; (6) an ellagic acid galloyl pentose; (7) an ellagic acid galloyl pentose or a dimethyl ellagic acid pentose; (8) a methyl ellagic acid galloyl pentose; (9) a methyl ellagic acid galloyl pentose; and (10) a methyl ellagic acid galloyl pentose.

Figure 1.

Figure 2.

Figure 3.

Figure 4.