In vitro Evaluation of Potential Anti-Inflammatory Agents from Peruvian Medicinal Plant Extracts

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ABSTRACT

The area of northern Peru is rich in plant biodiversity and is home to at least 510 medicinal native plant species, many of which are reported to have anti-inflammatory and cancer treating potential. Because of the positive medical implications anti-inflammatory therapeutics have on chronic inflammation and the progression of several types of cancers, this study assesses the anti-inflammatory effects of several Peruvian medicinal plant extracts on lipopolysaccharide (LPS)-induced RAW 264.7 murine macrophages. The *Lepechinia meyenii* ethyl acetate extract was the strongest suppressor of LPS-induced NF-κB transcription activity. A follow-up HPLC fractionation identified an active compound in this extract of atomic mass of 331 amu, however, the structure of this compound is yet to be determined. Furthermore, the *L. meyenii* active compound decreased LPS-induced iNOS and IL-6 mRNA levels dose-dependently. I demonstrate the presence of at least one potential anti-inflammatory compound isolated from *L. meyenii* which inhibits the expression of two pro-inflammatory proteins in LPS-induced murine macrophages. This study suggests that the downregulation of NF-κB transcription activity is one of the mechanisms of this anti-inflammatory response.

KEYWORDS

Lepechinia meyenii, NF-κB, RAW 264.7, iNOS, IL-6.

INTRODUCTION

Current cancer drug targets that have gained significant interest are mediators of inflammatory response, because inflammation has been associated as a key component of tumor progression (Coussens and Werb, 2002). The development of a variety of human cancers such as esophageal, stomach, colon, bladder, and prostate, in many cases, can be attributed to chronic inflammation (Hofseth and Ying, 2006). Therefore, novel anti-inflammatory agents can be effective in the treatment of these types of cancers.

A specific inflammation and cancer molecular drug target that has been the focus of numerous studies is the NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), which is a family of closely related transcription factors involved in inflammatory and innate immune responses present in every cell type (Prasad et al., 2009). NF-κB regulates the expression of genes involved in the development and progression of cancer such as proliferation, migration and apoptosis (Dolcet et al., 2005). For example NF-κB regulates the expression of IL-6, iNOS, COX-2 TNF-α, which are cytokines whose aberrant expression has been implicated in the pathogenesis of many diseases including cancer (Suh et al., 1998; Pan et al. 2009; Brichory et al., 2001; Lou et al., 2000; Arnott et al., 2002). For these reasons, NF-κB has become a high-throughput screening (HTS) drug target for cancer and inflammatory diseases. Despite the shifting attention towards synthetic small molecule HTS, natural sources such as plants are remain as great potential screening sources for cancer treating compounds due to their high diversity of complex secondary metabolites.

Plants possessing anti-cancer properties have played an enormous role in cancer treatment over the last thirty years and continue to hold great potential as sources for cancer treating compounds. According to the United States Food and Drug Administration, from 1981 to 2002, 62% of approved cancer drugs were of natural origin (Gonzales et al., 2006). In 2002, the bark derived compounds camptothecin and paclitaxel, accounted for about one third of the global anti-cancer drug market (Oberlines and Kroll, 2004). The vast structural diversity of phytochemicals has given plant derived cancer drugs a wide range of cancer treating actions (Cregg and Newman, 2005) and (Shoeb, 2006), and further underlines plants as promising candidates for drug sources.

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medicinal native plant species, many of them having anti-inflammatory and cancer treating potential (Bussmann and Sharon, 2006). The traditional use of medicinal plants in this area dates at least as far back as the Moche period (AD 100-800) (Bussmann and Sharon, 2006). The folklore of this area speaks of different plants used to treat illnesses such as hepatitis, internal inflammation, bronchitis, diabetes, tumors, and infections (Bussmann and Sharon. 2006) implying that these plants may possess medicinal compounds that can help attenuate these diseases. The long history of use and variety of illnesses treated by plants in this area suggests a wide chemical diversity of great medicinal potential, placing these plant species as promising drug sources. Yet despite the abundance of medicinal flora and traditional herbal medicinal usage, only a few studies have been conducted on a handful of species from this area to confirm the presence of cancer treating and anti-inflammatory compounds.

In this study, I test the following Peruvian medicinal plants for LPS-induced NF-κB activity suppression: *Lepechinia meyenii, Salvia discolor, Piper aduncum, Juglans neotropica, Cronquistianthus lavandulifolius, Borago officinalis, Rubus robustus, Cordia lutea*. I elucidate the most potent NF-κB inhibiting fraction from the *Lepechinia meyenii* ethyl acetate extract. I then test this active fraction for iNOS and IL-6 transcription inhibition. Finally, I attempt to elucidate one possible mechanism by which this active fraction inhibits NF-κB activity.

METHODS

Specimen collection

I purchased the following plants in the local market in Trujillo, Peru during the month of July 2010: *Lepechinia meyenii, Salvia discolor, Piper aduncum, Juglans neotropica, Cronquistianthus lavandulifolius, Borago officinalis, Rubus robustus, Cordia lutea*. I then verified the species of these plants by comparing them to deposited specimens in the Herberium of Antenor Orrego (HAO, Universidad Privada Antenor Orrego, Trujillo).

Plant extraction

I initially extracted the dried, pulverized plants in 250mL Erlenmeyer flasks with enough methanol to cover all of the plant material at room temperature for three days inside a hooded shaker. I then used a rotavapor to concentrate the extracts at 55°C. I resuspended each extract in 6mL of hot water and performed partitions with ethyl acetate and n-butanol giving three partitions per plant extract. I let the solvent for each extract evaporate at room temperature under a fume hood and weighed the resulting dried extract to find the concentration of each extract. I then dissolved each extract in varying amounts of DMSO depending on the dried weight to yield 20 mg/mL extracts.

Materials and cell cultures

RAW 264.7 cells, murinemacrophages, were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.). Dulbecco's modified Eagle's medium (DMEM), Dulbecco's phosphate buffer saline (D-PBS), lipopolysaccharide (E. coli, serotype 0127: B8; LPS), celastrol and dimethyl sulfoxidewere (DMSO) acquired from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Geneticin (antibiotic G-418) was purchased from Gibco BRL (Grand Island, NY, U.S.A.). All of the samples, solutions and buffers were prepared from deionized water. Primary antibodies for iNOS, IκBα, phospho-IκBα, and secondary antibodies were acquired from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Nitrocellulose membrane (NC membrane) was obtained from Whatman GmbH (Germany).

Cell culture and cell viability (MTT) assay

Murine leukemic monocytic macrophage cell line, RAW 264.7 cells were maintained and cultured at 37°C under humidified air, 5% CO_2 atmosphere in Dulbecco's Modified Eagle Medium (DMEM, GIBCO Invitrogen Corporation, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, 100 mg/mL streptomycin and 1.176 g/L sodium bicarbonate. I seeded the cells into 96-well plates at the density of 1×10^4 cells/well and allowed to adhere for 24 h, also at 37 °C under 5% CO_2. I treated the cells with plant extracts

(20µg/mL), *L. meyenii* and *S. discolor* ethyl acetate extract fractions (20µg/mL) for 18 h, then I added MTT solution to each well and incubated the plates for another 4 h at 37 °C. I removed the media after incubation and added DMSO to dissolve purple precipitates, which indicated the metabolic activity of viable cells. I used an Emax microplate reader (Molecular Devices, Sunnyvale, CA, U.S.A.) to read the optical density (O.D.) of the wells in the plates at 570 nm. I used Doxorubicin, a potent cytotoxic agent, as the positive control for cytotoxicity. High O.D. suggested high metabolic activity by viable cells, whereas low O.D. suggested low metabolic activity and therefore loss of cell viability.

NF-κB luciferase assay

I used the NF-κB luciferase reporter assay in LPS-induced mouse macrophage (RAW264.7) cells to determine NFkB activity suppression by the aforementioned samples according to the method of (Wu et al., 2010). Briefly, I plated stably transfected RAW264.7 cells with the NF-κB reporter gene in 96-well plates. Following a 24 h recovery period, I treated the cells with plant extracts (20µg/mL), *L. meyenii* and *S. discolor* ethyl acetate extract fractions (20µg/mL) for an additional 18 h in the presence of LPS (500 ng/mL). I used Celastrol, a potent anti-inflammatory, as the positive control for NF-κB expression inhibition. I used LPS dissolved in DMSO as the positive control for NF-κB expression. I used the Luciferase Reporter Assay System purchased from Promega (Madison, WI) to check for NF-κB luciferase activity. After treatment, I transferred 15 µL of the cell lysates from to opaque 96 well plates. Luciferase Assay Reagent (50 µL) was injected and read by a fluorometer (LMAX 2, Molecular devices).

Construction of peak library

Dr. Tyler Johnson (UC Santa Cruz, Philip Crews Lab) constructed peak libraries of both *L. meyenii* and *S. discolor* ethyl acetate extracts. Briefly, both extracts were analyzed through HPLC-MS-UV-ELSD and the separated fractions were decanted onto 96-well plates.

Cell culture and treatment for RT-PCR and Western blot

I plated RAW KB cells in clear Falcon 6-well plates using 2mL of RPMI 10% bovine serum media per well and incubated the plates for 18 hours to allow cell growth. I prepared three ten-fold serial dilutions of the *L. meyenii* active fraction from the initial 1 µg/mL concentration, which yielded four fractions of the following concentrations: 1 µg/mL, 0.1 µg/mL, 0.01 µg/mL and 0.001 µg/mL. I used DMSO as the treatment to show baseline transcription activity. To treat the cells, I added 1µL of sample and 1µL of LPS to each well except for the DMSO well. I incubated the plates for 18 hours at 37°C to allow the expression of immune response in the presence of the samples.

qPCR

I treated LPS-induced RAW 264.7 cells with the following *L. meyenii* active fraction concentrations: 0 µg/mL, 1 µg/mL, 0.1 µg/mL, 0.01 µg/mL and 0.001 µg/mL. I used DMSO with no LPS as the treatment to show baseline transcription activity of the proteins of interest. I treated the cells as described in the above sections. I extracted total RNA using RNeasy Micro Kit (Qiagen, Valencia, CA, USA) after 6 h of treatment. I determined RNA concentrations using the Quant-iTTM RiboGreen1 RNA Reagent and Kit (Invitrogen, Grand Island, NY, USA). I then reverse transcribed 0.1 mg of total RNA from each well sample to single-stranded cDNA by using TaqMan1 Reverse Transcription Reagents (catalog no. N808-0234, Applied Biosystems Inc., Foster City, CA, USA). I then performed qPCR analyses on 1µg aliquots of the cDNA preparations with SYBR Green PCR Master Mix (catalog no. 4309155, Applied Biosystems Inc., Foster City, CA, USA) to quantitatively detect the gene expression of, iNOS, IL-6, and glyceraldehydes 3-phosphate dehydrogenase (GAPDH) (as an internal standard) using Applied Biosystems 7900HT Fast Real-Time PCR System (Applied Biosystems Inc., Foster City, CA, USA). Integrated DNA Technologies Inc. (Coralville, IA, USA) designed the primer pairs by using Primer Quest Oligo Design and Analysis Tool. Melting curves confirmed the presence of single PCR. I carried out two independent experiments for each treatment.

Western blotting

I treated RAW 264.7 cells with of the *L. meyenii* active fraction using selected concentrations (0.1 µg/mL, 0.01 µg/mL and 0.001 µg/mL). I treated the cells similarly as described above. I lysed total cytoplasmic extracts after 6 h of treatment and carried out protein extractions. I ran 20 mg of protein samples through 10% gradient polyacrylamide gel (Criterion Tris–HCl gel, Bio-Rad Lab, Hercules, CA, USA) electrophoresis before transferring resolved proteins to polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore, Bedford, MA, USA) using a semi-dry transfer system (Fisher Scientific, Pittsburgh, PA, USA). I used 5% milk in 20 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, and 0.1% Tween 20 to block nonspecific binding of antibodies. Antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). I carried out immunodetection of total IKBα and pospho-IKBα proteins using respective primary antibodies (1:1000 in 5% BSA in PBST buffer) and horseradish peroxidase (HRP) conjugated secondary antibodies (1:3000 in 5% BSA in PBST buffer). I used the enhanced chemiluminescent system for detecting HRP on immunoblots (Thermo Scientific, Rockford, IL, USA) to determine the immunocomplexes and I used BioRad ChemiDoc XRS system (Hercules, CA, USA) to visualize and capture the bands. I used UN-SCANIT ™ software (Silk Scientific, UT, USA) to quantitatively determine the intensity of each band. The density ratio in the blot showed the relative intensity of each band against those of the controls in each experiment. Only one experiment was carried out for each treatment.

Statistical analysis

The results were presented as means ± standard error of the mean (SEM). Differences in mean values between groups in the iNOS qPCR results were analyzed by a one-way analysis of variance (ANOVA) using R Commander software. Student's t-test was used to analyze differences in mean values of the IL-6 qPCR results. The statistical significance of mean differences was based on a p value of <0.05.

RESULTS

NF-κB activity inhibition

From the initial 18 extracts tested for LPS-induced NF-κB activity inhibition, *Salvia discolor* and *Lepechinia meyenii* ethyl acetate extracts had the most potent inhibitory activity (Fig. 1). The cell viability test verified that these results were not false positives due to cytotoxicity (Fig. 2). Fractions H34, H35, H38 and H39 from the *L. meyenii* peak library potently inhibited NF-κB activity when compared to celastrol (Fig. 3). Fraction H28 from the *S. discolor* peak library was the only fraction to inhibit NF-κB activity (Fig. 4). Cell viability (MTT) assay verified that these results were not false positives due to cytotoxicity (data not shown).

iNOS transcript expression inhibition

The *L. meyenii* fraction H34 dose-dependently inhibited iNOS transcript expression. At a concentration of 0.001 µg/mL, the H34 fraction significantly inhibited iNOS transcript expression ($p=0.004$) when compared to the untreated LPS-induced group, however, here was no significance in the differences in iNOS transcript expression between all treated groups (Fig 5).

IL-6 transcript expression inhibition and Phospho-IκBα expression

The H34 fraction significantly inhibited IL-6 transcript expression ($p=0.025$) at 0.001 µg/mL while the three higher concentrations (0.01 µg/mL, 0.1 µg/mL, 1 µg/mL) reduced IL-6 transcript expression to undetectable levels (Fig. 6). There was no clear difference in the expression of phospho-IκBα between H34 treated groups (Fig 7).

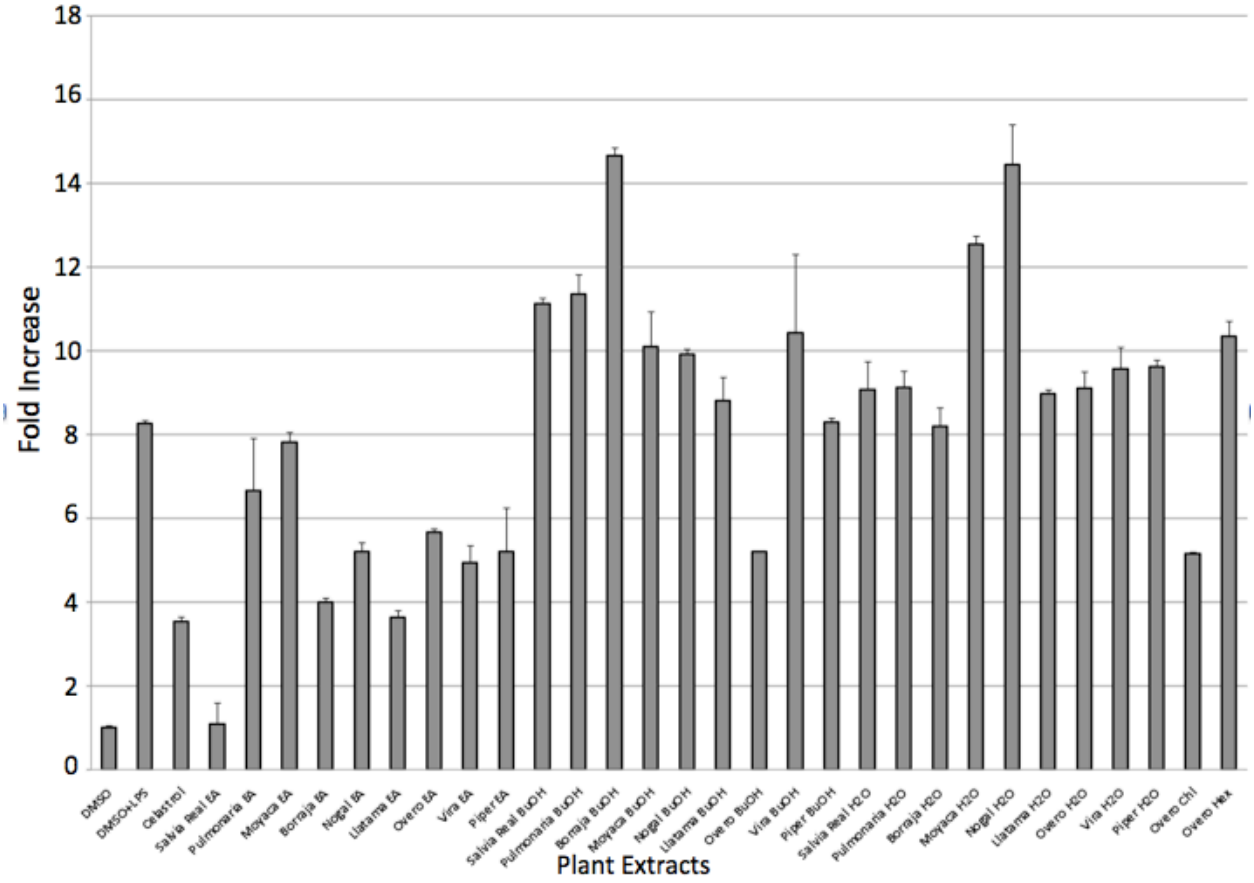

Figure 1: Effect of plant extracts on LPS-induced NF-κB luciferase expression in RAW 264.7 cells. Cells were cultured with plant extracts (20 µg/mL) in the presence of 100 ng/mL LPS for 18 h. Celastrol (25µM) was used as the positive control. Data are presented as mean ±SEM, n=2.

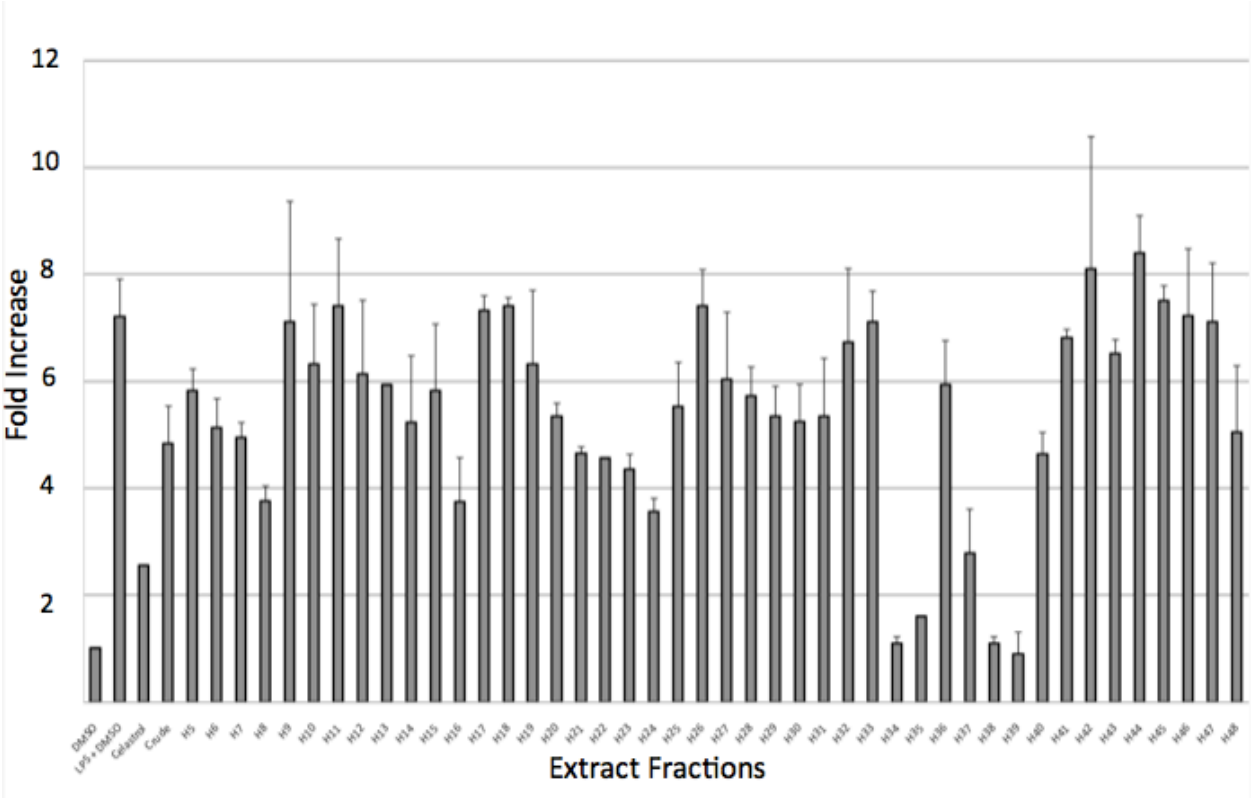

Figure 2: Effect of *L. meyenii* peak library fractions on LPS-induced NF-κB luciferase expression in RAW 264.7 cells. Cells were cultured with *L. meyenii* peak library fractions (20 µg/mL) in the presence of 100 ng/mL LPS for 18 h. Celastrol (25µM) was used as the positive control. Data are presented as mean ±SEM, n=2.

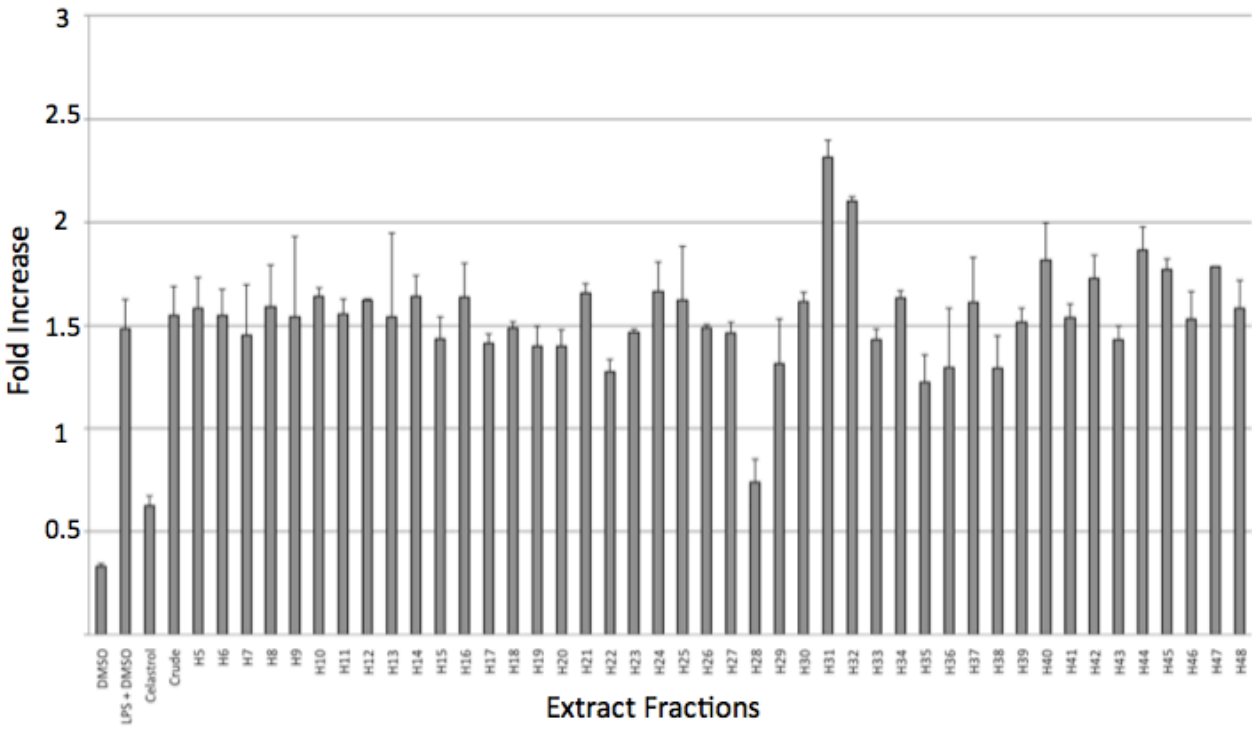

Figure 4: Effect of *S. discolor* peak library fractions on LPS-induced NF-κB luciferase expression in RAW 264.7 cells. Cells were cultured with *S. discolor* peak library fractions (20 µg/mL) in the presence of 100 ng/mL LPS for 18 h. Celastrol (25µM) was used as the positive control. Data are presented as mean ±SEM, n=2.

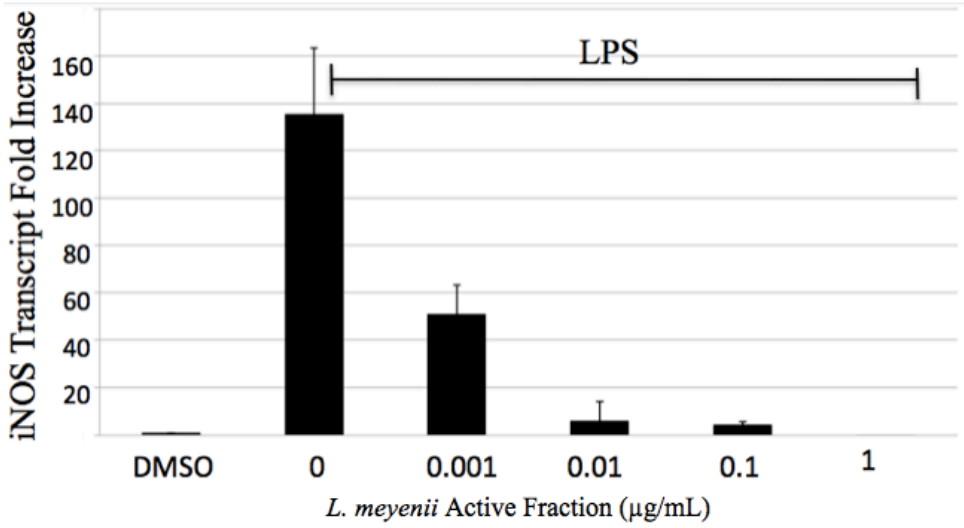

Figure 5: Effect of *L. meyenii* fraction H34 on iNOS transcript expression on LPS-induced RAW 264.7 cells. Cells were treated with increasing concentrations of the *L. meyenii* active fraction (H34) in the presence of 100 ng/mL LPS for 18 h. Each bar represents the mean ± SEM, n=2.

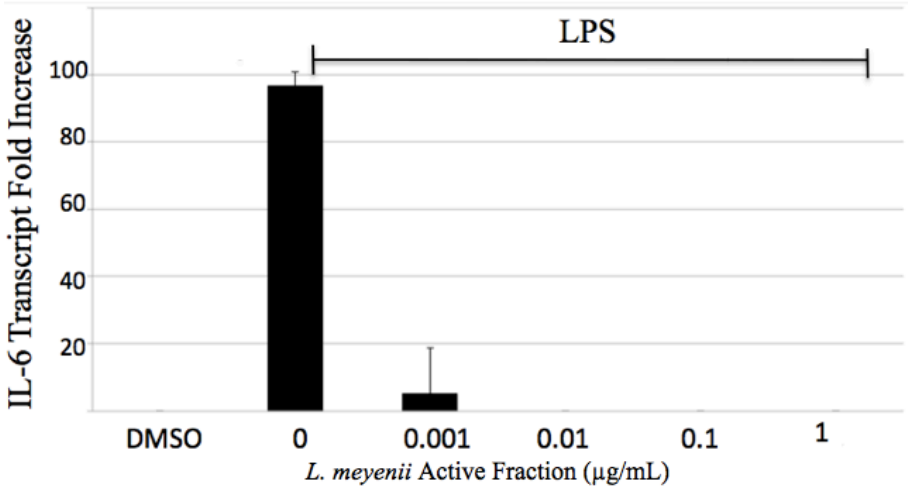

Figure 6: Effect of *L. meyenii* active fraction on IL-6 transcription expression on LPS-induced RAW KB cells. Cells were treated with increasing concentrations of the *L. meyenii* active fraction in the presence of 100 ng/mL LPS for 18 h. Each bar represents the mean ± SEM, n=2.

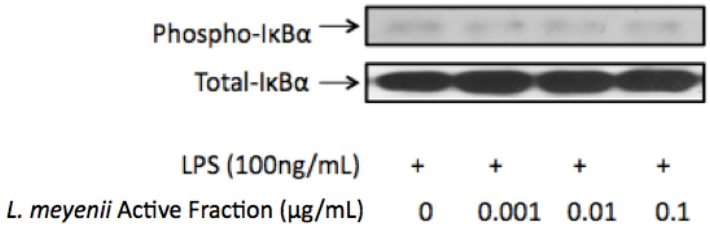

Figure 6: Effect of *L. meyenii* active fraction on Phospho-IκB expression on LPS-induced RAW KB cells. Cells were treated with increasing concentrations of the *L. meyenii* active fraction (H34) in the presence of 100 ng/mL LPS for 18 h. Photograph of the chemiluminescent detection of LPS-induced total and Phospho-IκBα expression blots representative of one experiment are shown.

DISCUSSION

In the present study, I examined fifteen Peruvian medicinal plants for suppression of LPS-induced NF-κB activity in murine macrophages. *L. meyenii* and *S. discolor* ethyl acetate extracts showed the most potent activity in the initial screening and the peak libraries of these extracts helped determine which fractions displayed this activity. All other plant extracts in the initial screen that did not inhibit NF-κB activity were not further studied. Although the *B. officinalis* ethyl acetate extract had similar activity as the S. discolor ethyl acetate extract, I was not able to do further studies on this plant during the time constraints of this project. The H34 *L. meyenii* fraction was the most potent NF-κB inhibitor so I performed further studies to verify its anti-inflammatory activity.

Inhibition of inflammatory response

The *L. meyenii* H34 fraction dose-dependently inhibited LPS-induced iNOS and IL-6 transcription levels in murine macrophages. High levels of IL-6, iNOS and other pro-inflammatory cytokines have been associated with chronic inflammatory diseases as well as various types of cancers thus making IL-6 and iNOS and other cytokines possible molecular targets for chemoprevention (Pan et al., 2009; Lou et al., 2000). IL-6 and iNOS promoters have numerous binding sites for transcription factors, which include NF-κB, AP-1, STATs and NFI-

L6 (Lee et al., 2003; Matasuka et al., 1993) however NF-κB is mainly responsible for the transcription of these cytokines by LPS stimulation (Xie et al., 1994). The *L. meyenii* H34 fraction significantly inhibited transcriptional activity of NF-κB in LPS-induced RAW 264.7 cells and therefore suggesting that the decrease in IL-6 and iNOS transcription can be attributed to the inhibition of NF-κB activity. NF-κB is normally found inactivated in the cytoplasm in complex with IκBα. NF-κB becomes free to translocate to the nucleus and upregulate pro-inflammatory genes when IκK phosphorylates IκBα into phospho-IκBα. The *L. meyenii* H34 fraction dose dependently decreased phospho-IκBα, and thus prevented the expression of IL-6 and iNOS by keeping NF-κB in its latent form attached to IκBα. Additional studies can test if and how IκK is affected by the H34 active fraction. Further studies are required to determine if the H34 fraction also inhibits other relevant transcription factors such as AP-1, STATs and NFI-L6.

Relevance of traditional usage

Traditionally, both *L. meyenii* and *S. discolor* have been used orally to treat inflammatory illnesses/discomforts. *Lepechinia meyenii* has been used to treat bronchitis and menstruation symptoms, whereas *S. discolor* has been used to treat cough symptoms and infections related to birth (Bussmann and Sharon, 2006), yet no studies have elucidated the mechanisms of action behind these reported uses. The results obtained for the *L. meyenii* H34 fraction support *L. meyenii's* traditional use as an anti-inflammatory herb, yet further studies are needed to fully validate this plant's medicinal usage. Interestingly, both of these plants have also been reported to have been used as memory aids (Bussmann and Sharon, 2006). There is a growing body of evidence, although conflicting, that suggests that a cause of Alzheimer's disease might be chronic neuroinflammation (Wyss-Coray 2006). There is research suggesting that non-steroidal anti-inflammatory drugs (NSAIDs) can have a promising effect on protection and treatment against Alzheimer's disease (McGeer and McGeer 2007). If further studies validate these two species as anti-inflammatory herbs, this information can suggest that these plants may also serve as neuro-protective herbs, although further progress must be made to establish inflammation as a cause for Alzheimer's disease.

Both *L. meyenii* and *S. discolor* are close relatives in the *Lamiaceae* family. The genera

Lepechinia, *Salvia*, and *Rosmarinus* belong to tribe *Mentheae*. *Rosmarinus officinalis*, most commonly known as the cooking sage Rosemary, is a member of this tribe and has been found to possess various anti-inflammatory compounds (Lai et al., 2009). *L. meyenii* and *S. discolor* share some of the compounds found in Rosemary, including two reported anti-inflammatory compounds Carnosic Acid and Rosmanol (Abreu et al., 2008; Bruno et al., 1991). Because the molecular weights of the compounds in the *L. meyenii* H34 fraction did not match with the molecular weights of Carnosic Acid and Rosmanol, that the compound responsible for the anti-inflammatory activity in this fraction is likely not either one of these compounds and can possibly be a novel compound.

Limitations

This study evaluated the anti-inflammatory activity of one HPLC fraction of the ethyl acetate *L. meyenii* extract. Time constraints did not allow for the evaluation of the other *L. meyenii* active fraction or the *S. discolor* active fraction. I was unable to purify the active compound in the H34 fraction, therefore it remains unclear whether the activity shown by this fraction is caused by the action of one single compound, or by the additive and/or synergistic effects of multiple compounds in this fraction. I was also unable to elucidate the structure of the active compound in the H34 fraction because of time limitations. I did not complete Q-PCR analyses of other pro-inflammatory such as TNF-α and COX-2 due to lack of reagents.

Future directions

Future studies should perform ^{1H}NMR, ^{12C}NMR and MS to elucidate the active compounds in the active fractions. Q-PCR studies can verify the effects of this fraction on the transcription of other relevant pro-inflammatory cytokines such as TNF-α, and COX-2. Further studies should also determine if the H34 fraction also inhibits other transcription factors involved in inflammatory action such as AP-1, STATs and NFI-L6. Based on the promising results of the potent iNOS and IL-6 dose dependant inhibition, possible future directions of this study would be *in vivo* testing on small mammals to verify the anti-inflammatory of these compounds in living organisms. Because both *L. meyenii* and *S. discolor* extracts significantly inhibited

inflammatory response, it would be interesting to assay other plants from the Lamiaceae family for anti-inflammatory activity.

CONCLUSION

The present study has identified *L. meyenii* and *S. discolor*, two medicinal plants traditionally used to treat illnesses and discomforts caused by inflammation with *in vitro* anti-inflammation activity in LPS-induced murine macrophages. This is the first study to give positive *in vitro* results of anti-inflammatory activity from both *L. meyenii* and *S. discolor*. The significant dose-dependant inhibition of iNOS and IL-6 transcript expression as well as the potent inhibition of NF-κB activity displayed by the *L. meyenii* H34 fraction indicates that *L. meyenii* possesses at least one anti-inflammatory compound that deserves further studies.

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