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Immunomodulatory Effects of Glycerol Echinacea purpurea Stem and Leaf Extract on RAW 264.7 Murine Macrophages

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Senior Honors Project

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Table of Contents

Abbreviation Key

ANOVA (Analysis of Variance)
ATCC (American Type Culture Collection)
CB_2 (Cannabinoid binding receptor 2)
CD# (Cluster of Differentiation molecule)
DMEM (Dulbecco's Modified Eagle Medium)
DPBS (Dulbecco's phosphate-buffered saline)
ELISA (Enzyme Linked Immunosorbent Assay)
EP (*Echinacea purpurea*)
EQ (Equation)
EtOH (Ethanol)
g (Gram)
GLY (Glycerol/Glycerin/Glycerine)
IL-# (Interleukin)
L (Liter)
LLC (Limited liability company)
LPS (Lipopolysaccharide)
ng (Nanogram)
MD-2 (Myeloid differentiation factor 2)
mL (Milliliter)
mM (Millimolar)
NO (Nitric oxide)
PRRs (Pattern Recognition Receptors)
rpm (Revolutions per minute)
SEM (Standard Error of the Mean; ± represents above or below)
SPSS (Statistical Product and Service Solutions)
TLR-# (Toll-like receptor)
TNF-alpha (Tumor Necrosis Factor-alpha; also written as TNF-α)
uL (Microliter; also written as µL)
uM (Micromolar; also written as µM)
URI (Upper Respiratory Tract Infection)

List of Figures

List of Tables

Abstract

The eastern and central North American flowering plant *Echinacea purpurea* (EP) is used by Native Americans as a traditional remedy for upper respiratory illnesses (URI). Active chemical constituents in EP include caffeic acid derivatives, alkylamides, and polysaccharides. The use of EP extracts have been suggested to shorten the duration of upper respiratory illness symptoms. Most studies suggest an immunomodulatory effect on innate immune responses. This research will test the immunomodulatory effects of a 50:50 glycerol:water extraction of EP stem and leaf on LPS-stimulated RAW 264.7 murine macrophages. Results will be compared to previous work using 75:25 ethanol root and 75:25 ethanol stem and leaf extract to determine differences in direct effect on RAW 264.7 cells. RAW 264.7 cells plated at 4x10^5 cells/well (500uL DMEM) in a 24-well plate are stimulated with bacterial lipopolysaccharide [LPS] (*E. coli* O55:B5) for 24 hours. Supernatants were reserved for cytokine TNF-alpha ELISA and samples were used to measure nitrites in solution by the Griess reaction to assess inflammatory responses. Preliminary experiments with extract diluted in DMEM at 15 uL/mL (0.75% glycerol), 30 uL/mL (1.5% glycerol) and 60 uL/mL (3% glycerol) showed inhibitory effect in cells stimulated by 100 ng/mL of LPS. Further experiments will examine lower concentrations of glycerol in extract in the culture environment to elucidate solvents effect on cell culture responses.

Keywords: Echinacea, Echinacea purpurea, RAW 264.7, immunomodulation, inflammatory response, herbal, extract

Introduction

I. Historic Usage of *Echinacea*

Historically, Native Americans of eastern and central North America utilized *Echinacea* species for pain relief, symptoms related to the common cold, wound treatment, and as an antidote to poisons and venoms (Borchers et al. 2000). Methods of Native American use for *Echinacea* included, consumption of juice or tea from the fresh flower, pulp from the roots, or smoking of the herb (Collins and Berkoff 1999). Settlers were introduced to *Echinacea* by the Native Americans, and at least one species, *Echinacea purpurea*, which was used by the Choctaw and Delaware Native Americans, was brought back to Europe (Borchers et al. 2000). While *Echinacea* usage fell out of physician practice in the United States, usage continued in Europe. Research in Germany has contributed greatly to the body of literature on *Echinacea* since 1992 (Collins and Berkoff 1999).

II. Active Components in *Echinacea*

Active chemical components of *Echinacea* include alkylamides (also called alkamides), phenolic compounds, and polysaccharides (Catanzaro et al. 2018). Alkylamides are unsaturated fatty acids that are present in *Echinacea* and other species, and the isobutylamide-type alkylamides are the main chemicals of this group found in *Echinacea* (Raduner et al. 2006). Phenolic compounds in *Echinacea* are mainly caffeic acid derivatives, of which caftaric, chicoric, cynarin, and chlorogenic acid are present from this group (Catanzaro et al. 2018).

Polysaccharides within *Echinacea* include arabinose, fructans, and arabinogalactans (Yang et al. 2018).

The diverse components of *Echinacea* are responsible for the variance and contradictory effects seen across studies with different extract preparations on macrophages. Isobutylamide-type alkylamides are responsible for inhibitory modulation of TNF-alpha production in LPS-stimulated macrophages, as well as stimulatory modulation of interleukin-10, suggesting an anti-inflammatory effect mediated by cannabinoid receptor type 2 (CB_2) (Raduner et al. 2006; Chicca et al. 2009). Phenolic caffeic acid derivatives such as chicoric acid have been found to significantly increase the transcription factor nuclear factor- kappa B when co-administered with 2,4-diene alkamide in T-cells (Manayi et al. 2015; Matthias et al. 2008). Additionally, a study by Bauer has suggested that EP's chicoric acid produces an anti-inflammatory effect by inhibiting anti-hyaluronidase (Bauer 1996). The polysaccharide arabinogalactan in EP has been suggested to activate macrophages due to a resemblance to bacterial lipopolysaccharide [LPS] (Catanzaro et al. 2018; Leuttig et al. 1989; Ren et al. 2023). The study by Ren et al. isolated an arabinogalactan from EP and found data to suggest that arabinogalactans are responsible for polarization of macrophages into the pro-inflammatory M1 phenotypes (2023). Studies by Bauer et al. and Borchers et al. have suggested that aqueous extracts of EP yield a chemical profile of polysaccharides, rather than ethanolic extracts (1988; 2000). TNF-alpha cytokine production and phagocytic activity in macrophages has also been observed to increase due to arabinogalactans in EP (Classen et al. 2006).

III. Previously Documented Effects

As previously mentioned, EP has several active components that have been examined for potential direct effects on the innate immune system (typically studied using macrophages). There have also been several *in vivo* studies that evaluate the effects of EP within the context of the complete immune system. The results of some of these studies add significance to the historical usage of EP as an alternative treatment, though, EP is now recommended as a complementary rather than standalone treatment (Collins and Berkoff 1999).

A review by Barrett has examined many previously documented effects such as reduction in contraction of URIs, reduction in cold and flu symptoms and severity, and reduction in duration of URI illness (2003). However, Barrett reports that many of the studies have significant limitations or confounds that skew any conclusions made. There are some consistent results reported in 10-40% reduction of symptoms during illness due to *Echinacea* (Barrett 2003). As a preventative measure, however, there are slight indications of prevention at 10-15% effect size (Barrett 2003). Barrett contends that if these mild to moderate benefits when taking *Echinacea* are real, then they are significant at a population level to combat discomfort and productivity loss (2003). In addition to URI, Collins and Berkoff mention that there have been studies conducted on *Echinacea* as a co-treatment to enhance antifungals for *Candida albicans* infections, as well as its wound-healing ability (1999). The results of these studies mirror Barrett's assessments, either not meeting scientific standards or not containing trial-blindness to remove potential bias (Collins and Berkoff 1999).

IV. Research Model

Cell Line. The immortalized RAW 264.7 cell line has been used as an immunological macrophage research model for at least 40 years. This cell line is derived from the BALB/c mice through transformation via the Abelson Leukemia virus (Raschke et al. 1978; Taciak et al. 2018). RAW 264.7 macrophages feature an increased production of nitric oxide (NO) in response to LPS stimulation (Raschke et al. 1978; Taciak et al. 2018). According to the study of Taciak et al., RAW 264.7 macrophages produce stable NO response and phagocytic activity from passages 10 to 30 (Taciak et al. 2018). This 5 to 15 week experimental window makes them a valuable model for inflammatory research in addition to their responsiveness.

Stimulatory Agent. For assessing the immunomodulatory capacity of glycerol EP stem and leaf extract, NO production upon LPS stimulation will be measured. LPS is present on the outer surface of gram-negative bacteria (Farhana and Khan 2023). These components of the bacterial membrane act as a barrier to harmful molecules for bacteria. LPS is comprised of three groups; Lipid A, which is the endotoxic aspect that will be recognized by macrophages, the O-antigen, which is responsible for serotype distinction across species, and a hydrophilic polysaccharide core (Figure 1) (Farhana and Khan 2023).

Specifically, LPS stimulates macrophages through lipid A interacting with the CD14, TLR-4, MD-2 complex in macrophages (Meng and Lowell 1997; Park and Lee 2013). The presence of serum LPS-binding protein enhances the binding of LPS to macrophage pattern recognition receptors (PRRs) (Meng and Lowell 1997). In a complete immune system context, the O-antigen of LPS is antigenic (Farhana and Khan 2023), allowing specific serotype targeting via cytotoxic effector cells. LPS is a potent stimulator to macrophages (RAW 264.7 cells in

particular) and has a relevant role in many pathological inflammatory responses, making this stimulatory agent valuable for the purposes of this research.

Measured Agent. Macrophages, upon stimulation with LPS, will release several inflammatory mediators in addition to their enhanced phagocytic capacity. An integral mediator that contributes to macrophage cytotoxicity is NO (Coligan et al. 1991). NO is considered a reactive nitrogen species, contributing to its antimicrobial ability via electron scavenging potential within the context of infection (Green and Nacy 1993). Additionally, NO potentiates inflammation via vasodilation, increasing blood flow near site of infection (Green and Nacy 1993). Due to its reactivity, NO is a transient molecule, quickly breaking down into more stable molecules of nitrites and nitrates. Utilizing this transience, a relatively inexpensive colorimetric analysis using spectrophotometry can be performed to measure the stable end products as an indirect quantitation of NO. Since the stimulated macrophages will be releasing NO into supernatant, Coligan et al. describes a reliable protocol for measuring nitrites in solution using a surrogate marker (1991). This marker is made using the Griess reagent, which will be added to supernatant samples before being analyzed by spectrophotometry at 550 nm. The reaction is comparatively faster and more straightforward than assays for measurement of other inflammatory mediators, making NO a primary target for this project.

Griess Reaction. The specific mechanism that allows for colorimetric analysis of nitrites in supernatant is by utilizing these nitrites and an acidic environment, conferred by phosphoric acid, to convert sulfanilamide and N-(1-Naphthyl)ethylenediamine dihydrochloride into an azo dye via a diazotization reaction; this dye is what is measured with absorbance values ranging from 520 nm to 550 nm (Promega Corp. 2009). 550 nm is closer to the peak of the absorbance curve of the azo dye (540 nm), which is preferred (Bryan and Grisham 2007).

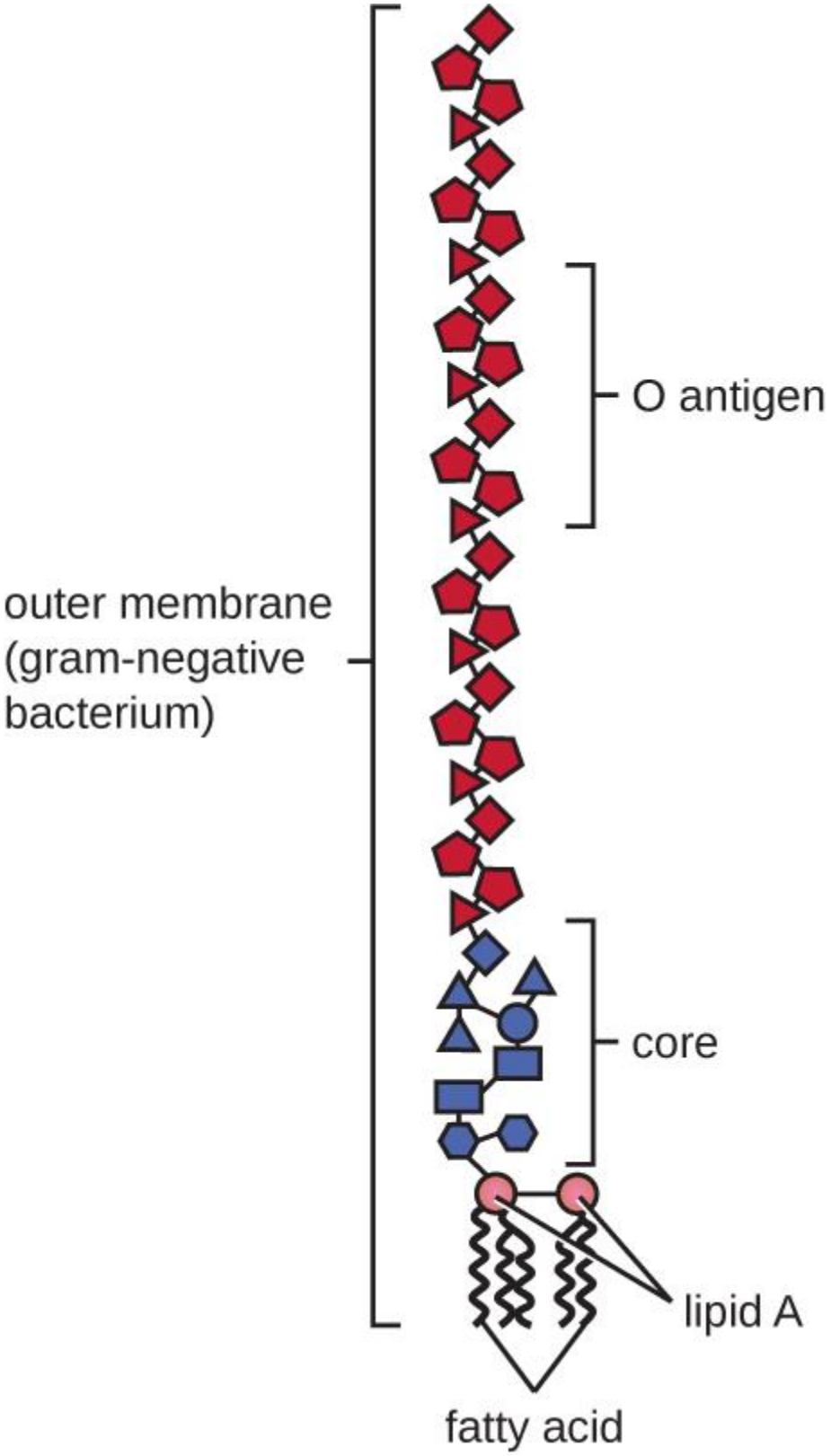

Figure 1. LPS structure; lipid A, polysaccharide core, and O-antigen. Wikimedia Commons.

V. Research Objectives

The objectives for this study are to evaluate immunomodulatory effects of GLY:EP on RAW 264.7 macrophages primarily. EtOH:EP extracts are also evaluated. We have chosen to measure NO production through nitrites in supernatant. A dose-dependent increase in nitrites in supernatant will suggest GLY:EP as pro-inflammatory, whereas a dose-dependent decrease in nitrites in supernatant will suggest GLY:EP as anti-inflammatory in RAW 264.7 macrophages. An observation of a dose-dependent decrease in nitrites in supernatant is expected when considering the literature discussing active components in EP.

Materials and Methods

I. Cell Culture

RAW 264.7 macrophages (ATCC) are cultured in high glucose complete DMEM (Lonza Group Ltd.) with 4mM L-glutamine and 4.5 g/L glucose and supplemented with 1.5 g/L sodium bicarbonate (Sigma-Aldrich), 100 IU penicillin/ 100 mg Streptomycin (MP Biomedical, LLC.) and 10% fetal bovine serum (Atlanta Biologicals). They are maintained with passage primarily by scraping every 4 days in $25cm^2$ flasks kept at 37°C in 5% CO_2 and ~90% humidity. Trypsinization was used in cell passage, although limited in its use. Concerning passage scheduling, it should be noted that upon examining data from cells, 4-day passage cycles began to yield cells that were in stationary phase, causing inconsistencies in cellular response. Therefore, the passage cycle was amended to every 3 days to produce more consistent cellular response in growth phase for assays.

Trypsinization Protocol. Cells were washed once with 5 mL of Dulbecco's phosphate-buffered saline (DPBS) to remove any leftover serum that would inactivate the trypsin. DPBS was decanted and a 3 mL aliquot of 0.25% trypsin was added to the flask. The trypsin was left to sit for 5 minutes at room temperature for adequate digestion of adhesion proteins. Cells were mechanically dislodged against a hard surface and the flask was gently flushed with 1 mL of DMEM. Then, 10 mL of fresh DMEM complete was added, as well as flask contents, to a 50 mL conical to be centrifuged (Eppendorf centrifuge 5810R, 15 AMP. Ver. 5811F; no. 0036920). Centrifuged for 5 minutes at room temperature at 1000 rpm with counterweight. New supernatant was decanted into waste container and 1 mL of DMEM complete was gently added to the centrifuged conical. The cell pellet was gently triturated for resuspension. Care was taken not to bubble by slowly bringing volume up to 10 mL of DMEM complete for complete resuspension protocol.

II. Preparations and Treatment

Initially, one gram of dried EP stem and leaf powder (ChromaDex) was mixed in 5mL of 75:25 EtOH:H_2O solvent using a 15 mL conical tube and vortexed for at least 10 minutes. The mixture sat at room temperature overnight before use and was kept refrigerated at -20 °C afterwards. The resulting color of this extract after 24 hours was light green.

One gram of dried EP stem and leaf powder (ChromaDex) was mixed in 10 mL of 50:50 GLY: H_2O solvent using a 15 mL conical tube and vortexed for at least 10 minutes. The mixture sat at room temperature overnight before use and was kept refrigerated at -20 °C afterwards. The solvent was very viscous upon putting the EP powder into the tube. When mixing through

vortex, inverting the tube occasionally helped the powder distribute evenly throughout the solvent. The resulting color of this extract after 24 hours was a brownish-yellow, not unlike honey.

Standard Procedure. RAW 264.7 cells were scraped and seeded in a 24-well plate at 4x10^5 cells/well in complete DMEM (high glucose variant) and left to adhere for 12 hours. Immediately preceding treatment, 500 uL of complete DMEM was added to wells and the resulting volume was aspirated using a glass pipette connected to the waste flask and pump. This wash step was repeated twice to remove any remaining non-adhered cells before dispensing 500 uL of treatment into the empty wells. A serial dilution of a 5 mg LPS (*E. coli* O55:B5, Sigma-Aldrich) stock was used to reach experiment concentrations. Experiments were considered within the context of 0 ng/mL LPS negative control (500 uL of complete DMEM only) and 100 ng/mL LPS positive control, as well as a comparison response between 0 and 100 ng/mL (typically 10 ng/mL LPS). Extract treatments contained 100 ng/mL LPS to assess immunomodulatory effects within the context of a stimulated cell. A parallel experiment was conducted alongside any treatment with EP to assess the effects of the solvent alone on the response of the stimulated cells. Treatments are analyzed using spectrophotometry for nitrite presence after 24 hours of incubation in cell culture conditions (Coligan et al. 1991; Green and Nacy 1993). Colorimetric distinctions dependent on analyte concentrations are produced by Griess reaction (Coligan et al. 1991).

Ethanolic EP Treatments. Four treatments of 100 ng/mL LPS + EP (0.375%, 0.75%, 1.5% and 3% EtOH:EP extract) were added to adhered and washed RAW 264.7 cells in quadruplicate. A parallel treatment of 100 ng/mL LPS + 0.375%, 0.75%, 1.5% and 3% EtOH

without EP was given to assess the effect of ethanol alone on NO response (treatments were in quadruplicate, except for 0.375% which was repeated in triplicate).

Treatments used an EtOH:H_2O 75:25 solvent, where percentages of solvent were calculated using this equation (EQ 1);

$$\frac{\left(\frac{X\%}{100} \times 1000\text{uL}\right)}{S\%}$$

Where X% represents the desired percentage of solvent in treatment and S% represents the mix ratio of the solvent used. The equation yields volume of extract in microliters per 1 mL for desired percentage of solvent in treatment.

EtOH:EP treatments/extract solvent volumes: 0.375% (5 uL/mL), 0.75% (10 uL/mL), 1.5% (20 uL/mL) and 3% (40 uL/mL).

Glycerol EP Treatments. Five treatments of 100 ng/mL LPS + EP (0.375%, 0.5%, 0.75%, 1.5% and 3% GLY:EP extract) were added to adhered and washed RAW 264.7 cells in quadruplicate. A parallel treatment of 100 ng/mL LPS + 0.375%, 0.5%, 0.75%, 1.5% and 3% GLY without EP was given to assess the effect of glycerol alone on nitric oxide response (treatments in quadruplicate).

Treatments used a GLY:H_2O 50:50 solvent, where percentages of solvent were calculated using equation 1. GLY:EP treatments/extract volumes: 0.375% (7.5 uL/mL), 0.5% (10 uL/mL), 0.75% (15 uL/mL), 1.5% (30 uL/mL) and 3% (60 uL/mL).

It is important to note that this GLY:EP extract requires refining. The original extract in 10 mL of solvent is not accessible immediately, as the plant matter caused it to coagulate. Centrifuging the extract allowed for better access of the crude extract. However, it was too thick for a standard P1000 pipettor tip to function properly. Cutting off the edge off the tip allowed for a sufficiently large diameter for the pipette to aspirate the crude extract. The crude extract was

dispensed in 200 uL increments into 1 mL sample tubes to hold, centrifuged, and vortexed for working extract.

III. Nitric Oxide Analysis

The 24-well plate was centrifuged at 1000rpm for 2 minutes. A 50 uL supernatant sample from each well is used for analysis. A 100 uL volume of Griess reagent (50:50 mix of 1% sulfanilamide (Sigma-Aldrich) and 0.1% N-(1-Naphthyl)ethylenediamine dihydrochloride (Sigma-Aldrich) both in 2.5% phosphoric acid (Sigma-Aldrich) were added to samples, and nitrite presence was determined by spectrophotometry at 550 nm (μQuant, Biotek Instruments Inc.). NO production can then be indirectly measured through quantitation of nitrites in supernatant. Absorbance values are compared to a sodium nitrite (Sigma-Aldrich) standard curve (0, 1, 5, 10, 25, 50, 75, 125 uM) to determine nitrite concentrations.

IV. Statistical Analysis

Statistical Product and Service Solutions (SPSS) Ver. 28 (Chicago, IL), was used to perform statistical comparisons for experimental results. Treatments were evaluated using a one-way analysis of variance (ANOVA) mean comparison with a post-hoc Dunnett's 2-sided T-test. A P-value of ≤ 0.05 indicates a statistically significant difference. Treatments were compared to both the positive (100 ng/mL LPS) and negative control (0 ng/mL LPS). The analyses tables can be found in appendix A.

Results

I. Ethanol EP Treatments on NO Response

Figure 2. Our first experiment utilizing our EtOH:H_2O 75:25 extracts yielded mixed results. The solvent concentrations in the extracts were 0.15%, 0.75%, and 1.5%. The EtOH:EP extract for this experiment was inconsistent and not statistically different when compared to the positive control. The EtOH control showed a minor dose dependent trend in the visible data presentation, but was not statistically different from the positive control either. There was no discernable effect in the extracts or solvent controls.

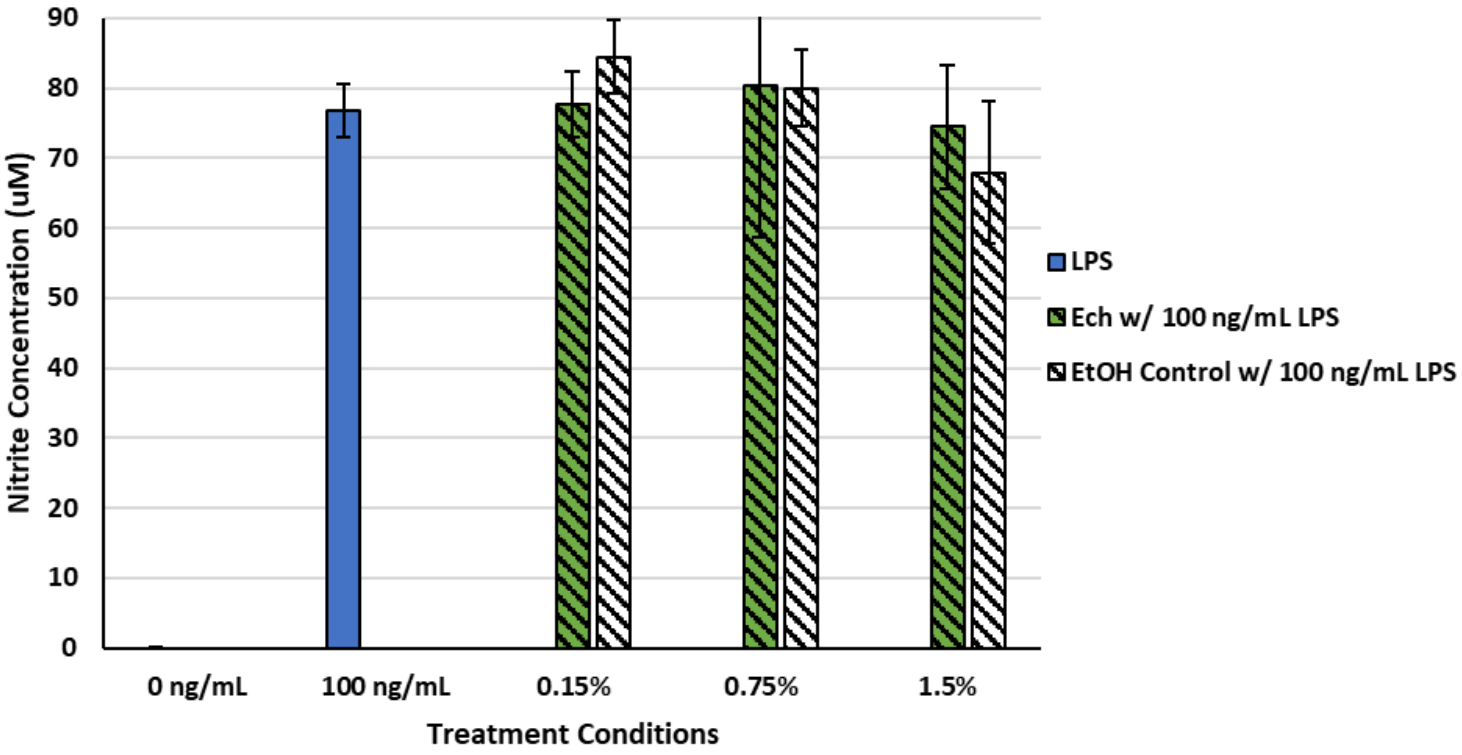

Figure 2. EtOH Exp. 1, Pass 9. Comparison of 75:25 Ethanol *E. purpurea* Stem and Leaf Extract v. 75:25 Ethanol Control at 24 hours. Significance at $p \leq 0.05$ when compared to 100 ng/mL LPS. Each bar represents the mean of trials in quadruplicate, with the error bars representing the ±SEM across the four trials (n = 4).

Figure 3. Experiment 2 utilizing EtOH EP extracts yielded some statistically different results in comparison to the positive control. This experiment used 0.375%, 0.75%, 1.5%, and 3% solvent concentrations. Statistical analysis indicates statistically different concentrations of nitrites in supernatant across all treatment groups of the EP extract. For the EtOH control, only 1.5% and 3% were indicated as statistically different. The data for this experiment suggests a statistically significant dose dependent reduction of nitrites in supernatant in stimulated RAW 264.7 macrophages when treated with 75:25 EtOH:EP extract at 0.375%, 0.75%, 1.5%, and 3% solvent concentrations. Only 1.5% and 3% EtOH control concentrations reduced NO in supernatant significantly when compared to positive control.

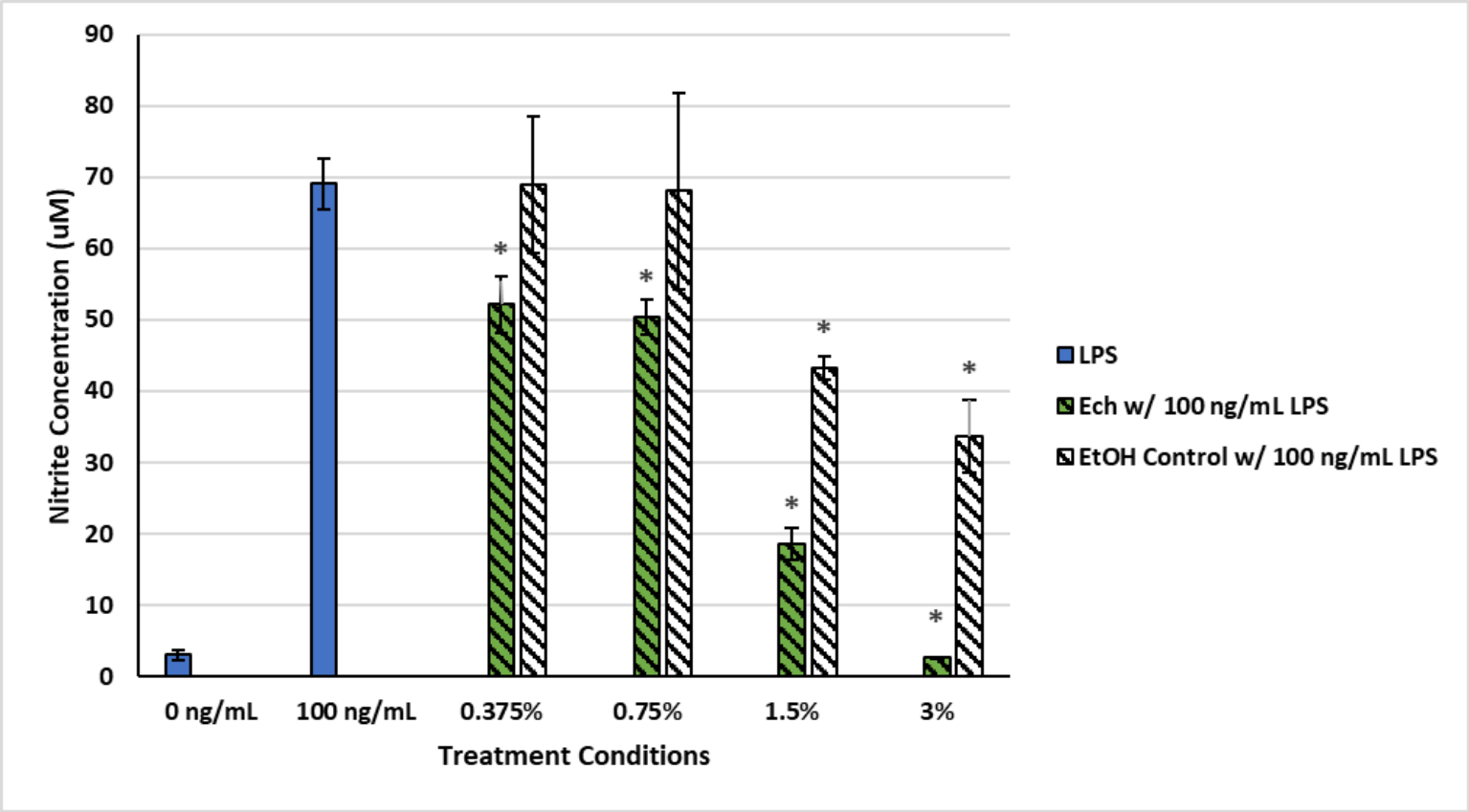

Figure 3. EtOH Exp. 2, Pass 14. Comparison of 75:25 Ethanol *E. purpurea* Stem and Leaf Extract v. 75:25 Ethanol Control at 24 hours. Significance at $p \leq 0.05$ when compared to 100 ng/mL LPS. Each bar (except 0.375% EtOH control) represents the mean of trials in quadruplicate, with the error bars representing the ±SEM across the four trials (n = 4). The 0.375% EtOH control bar represents the mean of trials in triplicate, with the error bars representing ±SEM across the three trials (n = 3).

Figure 4. Experiment 3 utilizing EtOH:EP extracts yielded inconsistent results. This experiment used 0.15%, 0.75% and 1.5% solvent concentrations in the treatments. The significance of the treatments in this experiment mirrors figure 2 with no statistically significant data points in both EtOH:EP extracts or EtOH controls when compared to the positive control. Despite the 1.5% EtOH control looking visibly different from the positive control, the p-value indicated in appendix A is 0.772, which is not statistically significant ($p \leq 0.05$).

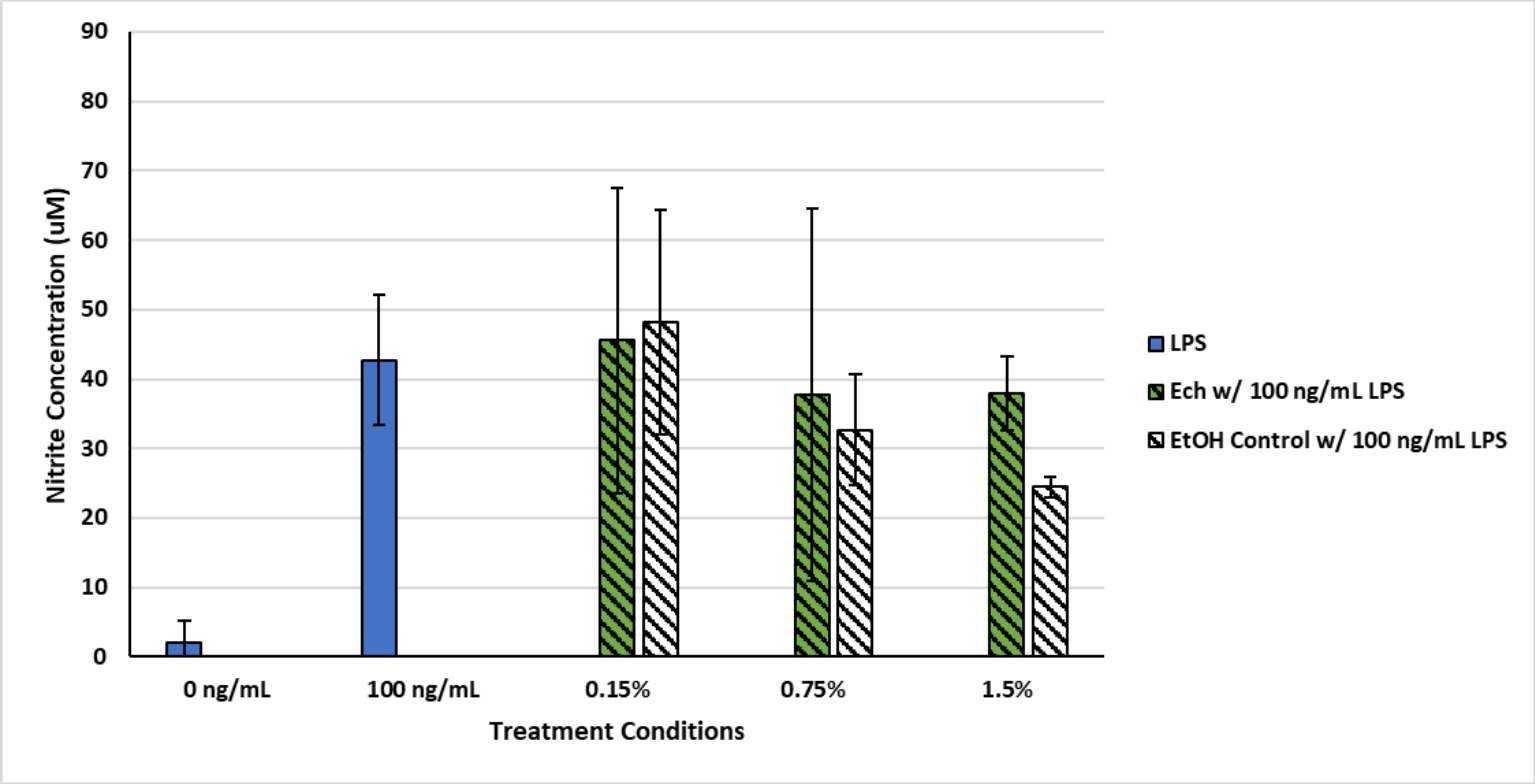

Figure 4. EtOH Exp. 3, Pass 6. Comparison of 75:25 Ethanol *E. purpurea* Stem and Leaf Extract v. 75:25 Ethanol Control at 24 hours. Significance at $p \leq 0.05$ when compared to 100 ng/mL LPS. Each bar represents the mean of trials in quadruplicate, with the error bars representing the ±SEM across the four trials (n = 4).

II. Glycerol EP Treatments on NO response

Figure 5. Experiment 4, utilizing GLY:EP extracts yielded consistent results. This experiment used 0.75%, 1.5%, and 3% solvent concentrations in treatments. Statistical analysis indicated all three EP extracts as statistically different compared to the positive control, and the GLY controls of 0.75% and 3% as statistically different when compared to the positive control. The data for this experiment suggests a statistically significant dose dependent reduction of nitrite in supernatant in stimulated RAW 264.7 macrophages when treated with 50:50 GLY:EP extract at 0.75%, 1.5%, and 3% solvent concentrations. The solvent control seems to have an independent effect of some kind, but the statistical analysis did not indicate a uniform dose dependent decrease in supernatant nitrite.

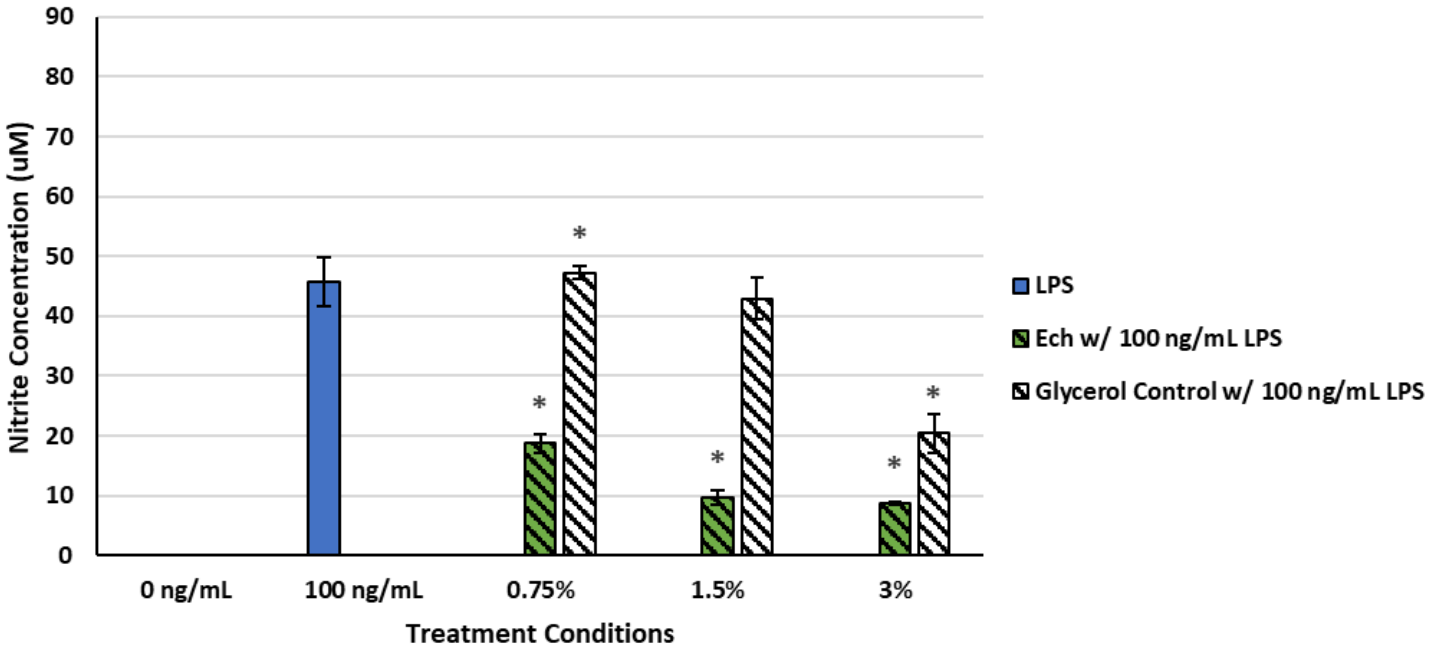

Figure 5. GLY Exp. 1, Pass 9. Comparison of 50:50 Glycerol *E. purpurea* Stem and Leaf Extract v. 50:50 Glycerol Control at 24 hours. Significance at $p \leq 0.05$ when compared to 100 ng/mL LPS. Each bar represents the mean of trials in quadruplicate, with the error bars representing the ±SEM across the four trials (n = 4).

Figure 6. Experiment 5, utilizing GLY:EP extracts yielded inconsistent results. This experiment used 0.375%, 0.5%, and 0.75% solvent concentrations in treatments. In this experiment, there was an EP group with no stimulatory agent added in addition to a solvent control. Statistical analysis indicates no statistical difference for all treatment groups and the solvent control groups when compared to the positive control. Additionally, when compared to the negative control, the EP alone (0 ng/mL LPS) was not statistically different. The data for this experiment indicated no dose dependent effects on stimulated RAW 264.7 macrophages when treated with 0.375%, 0.5%, and 0.75% solvent concentrations.

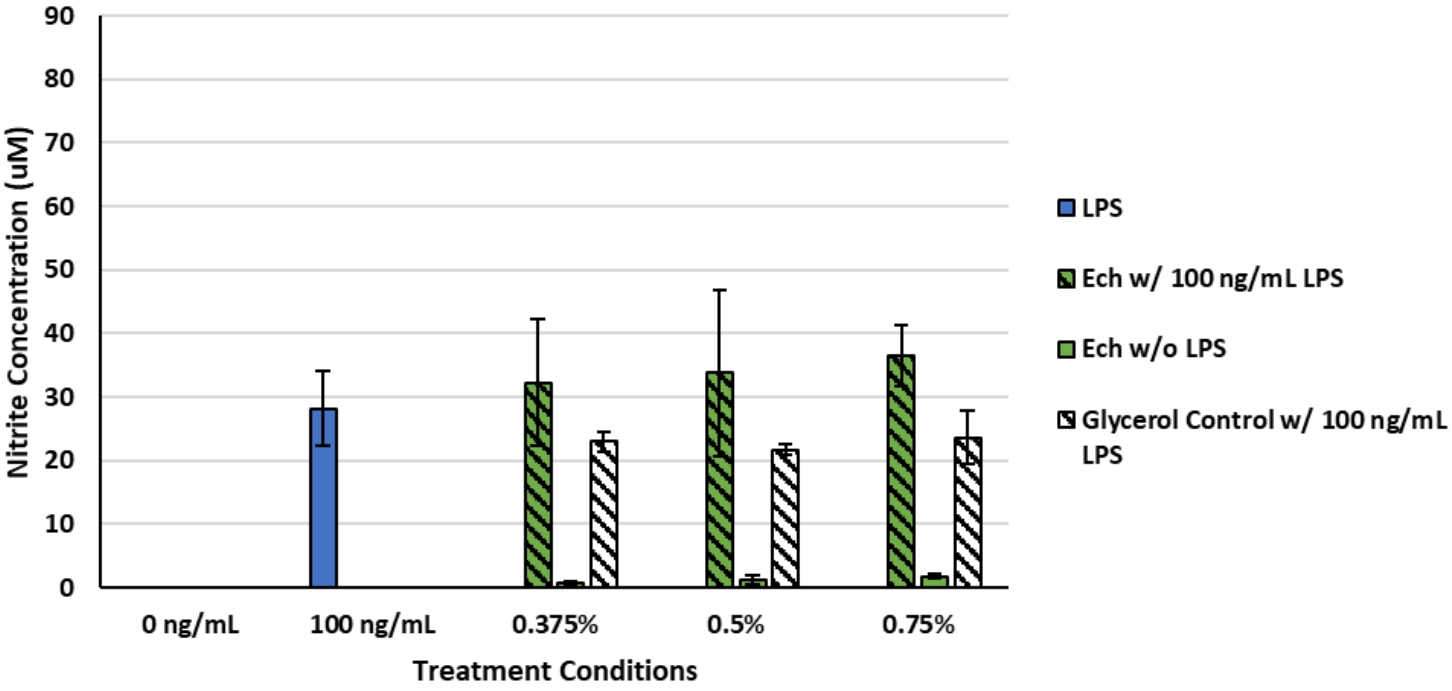

Figure 6. GLY Exp. 2, Pass 11. Comparison of 50:50 Glycerol *E. purpurea* Stem and Leaf Extract v. 50:50 Glycerol Control at 24 hours. Significance at $p \leq 0.05$ when compared to 100 ng/mL LPS. Each bar represents the mean of trials in quadruplicate, with the error bars representing the ±SEM across the four trials (n = 4).

Figure 7. Experiment 6, utilizing GLY:EP extracts yielded consistent results. This experiment used 0.375%, 0.75%, and 1.5% solvent concentrations in treatments. Like with figure 6, EP with no stimulatory agent served as treatment group. Statistical analysis indicates a statistical difference for all treatment groups and all solvent control groups when compared to the positive control. There is no statistical difference for the EP alone (0 ng/mL LPS) when comparing to the negative control. The data for this experiment suggests a dose dependent decrease of nitrites in supernatant when stimulated RAW 264.7 macrophages are treated with 0.375%, 0.75%, and 1.5% glycerol EP. Additionally, the 0.375%, 0.75%, and 1.5% glycerol controls seem to have an independent effect on stimulated RAW 264.7 macrophages that is statistically different when compared to the positive control.

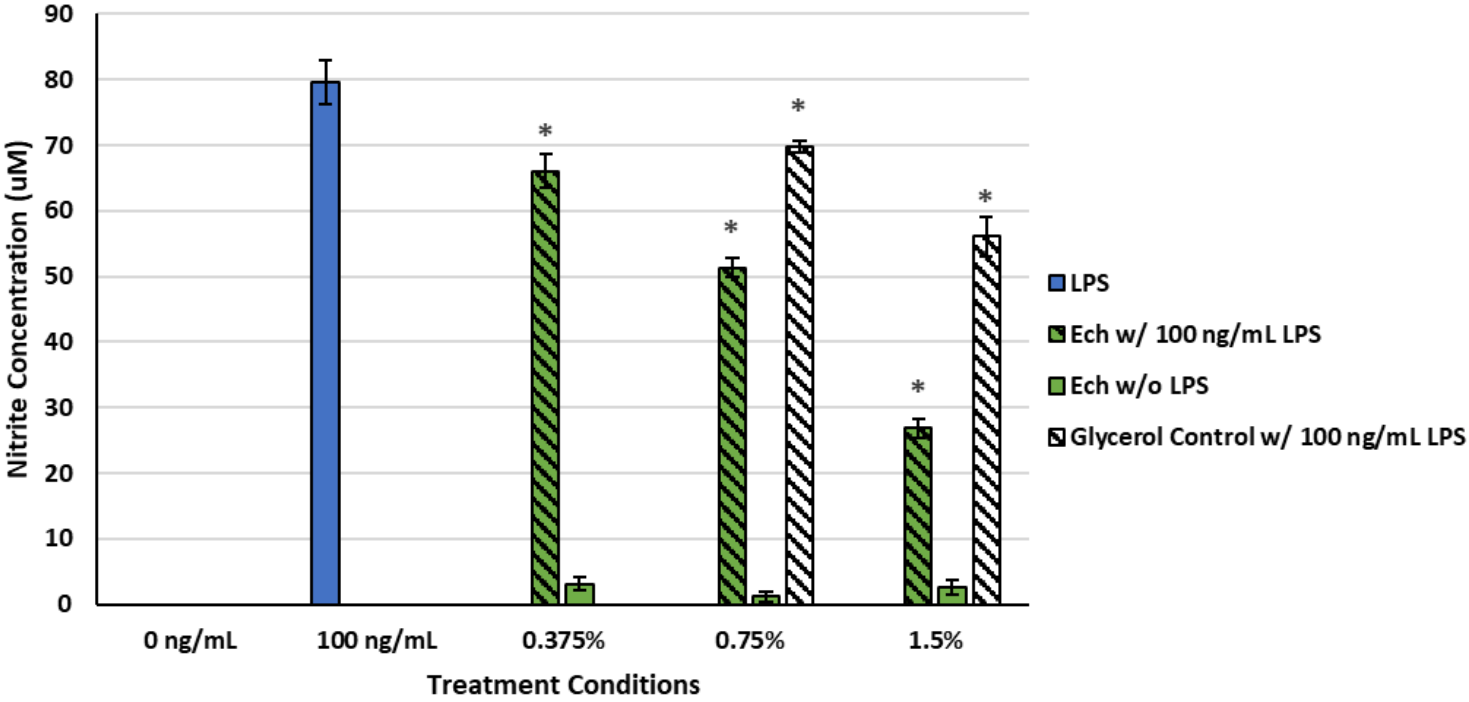

Figure 7. GLY Exp. 3, Pass 13. Comparison of 50:50 Glycerol *E. purpurea* Stem and Leaf Extract v. 50:50 Glycerol Control at 24 hours. Significance at $p \leq 0.05$ when compared to 100 ng/mL LPS. Each bar represents the mean of trials in quadruplicate, with the error bars representing the ±SEM across the four trials (n = 4).

Figure 8. The 0 ng/mL (8a) and 100 ng/mL (8b) controls showcase the inactivated and activated states of the RAW 264.7 macrophages. The 1.5% glycerol control + 100 ng/mL LPS (8c) does not appear different from the positive control, featuring enlargement and granules as typical of an activated macrophage. The 1.5% GLY:EP extract (8d), however, appears to have a comparatively high proportion of macrophages that are considerably larger with more granules visible when compared to the controls.

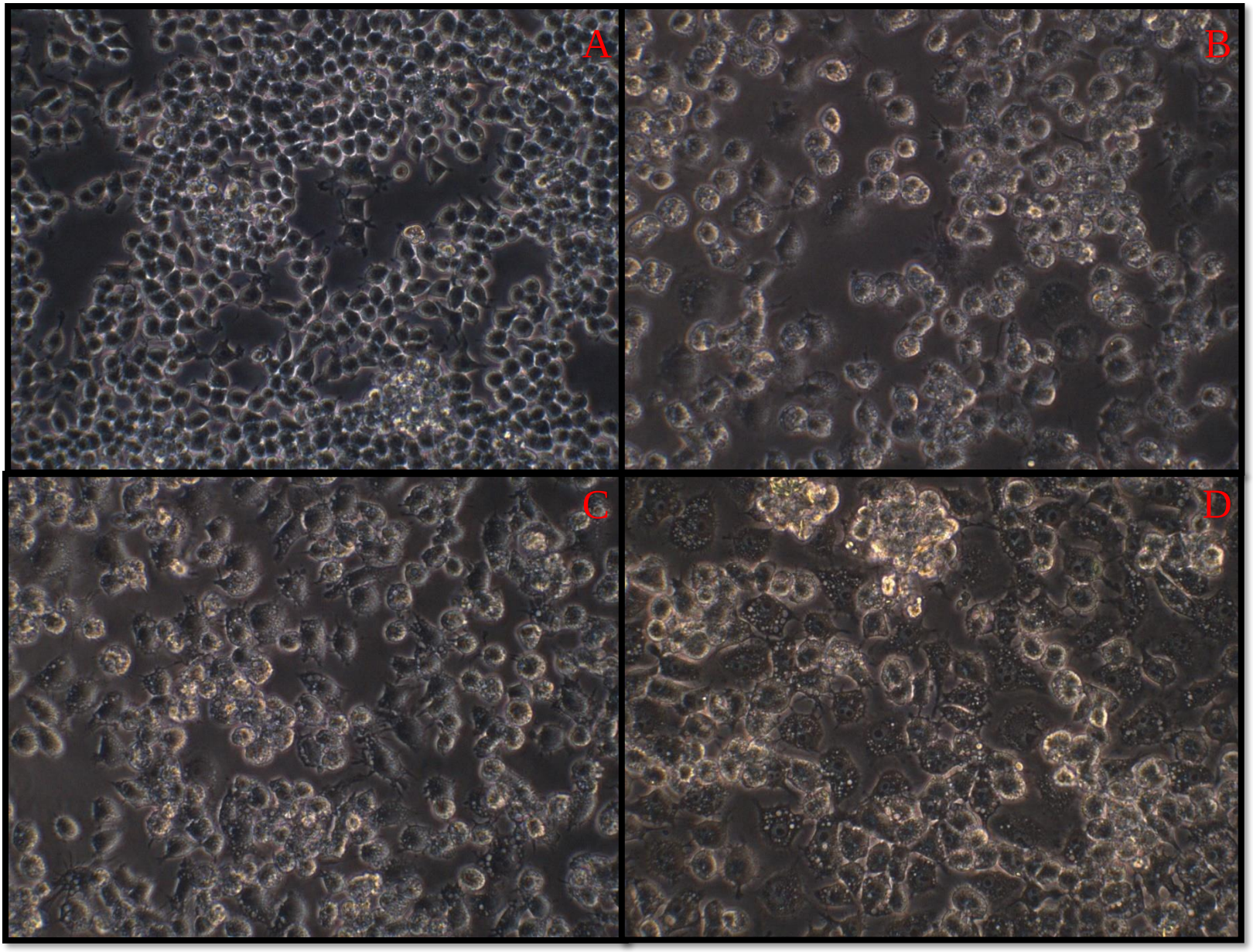

Figure 8. Photographs of RAW 264.7 macrophages with controls and treatment. ***A:*** RAW 264.7 cells with 0 ng/mL LPS. ***B:*** RAW 264.7 cells with 100 ng/mL LPS. ***C:*** RAW 264.7 cells with 1.5% glycerol control + 100 ng/mL LPS. ***D:*** RAW 264.7 cells with 1.5% GLY:EP extract + 100 ng/mL LPS. Images captured at 400x magnification using an Olympus B202 inverted microscope and OPELCO (optical elements corporation) software.

Figure 9. The 0 ng/mL (9a) control showcases the inactivated state of the RAW 264.7 macrophages. The 0.375% GLY:EP without LPS (9b) does not appear to have significant differences in nitrite concentration (figure 6) when compared to the negative control, but it does appear visually different when viewed under the microscope at 400x magnification. The negative control has no granular macrophages and minimal extension of cell membrane, however the 0.375% GLY:EP without LPS treatment has a small number of granular macrophages in addition to extended macrophages that appear to be interacting with the environment. The 0.5% GLY:EP (9c) and 0.75% GLY:EP (9d) without LPS treatments also show a small number of granular and extended macrophages.

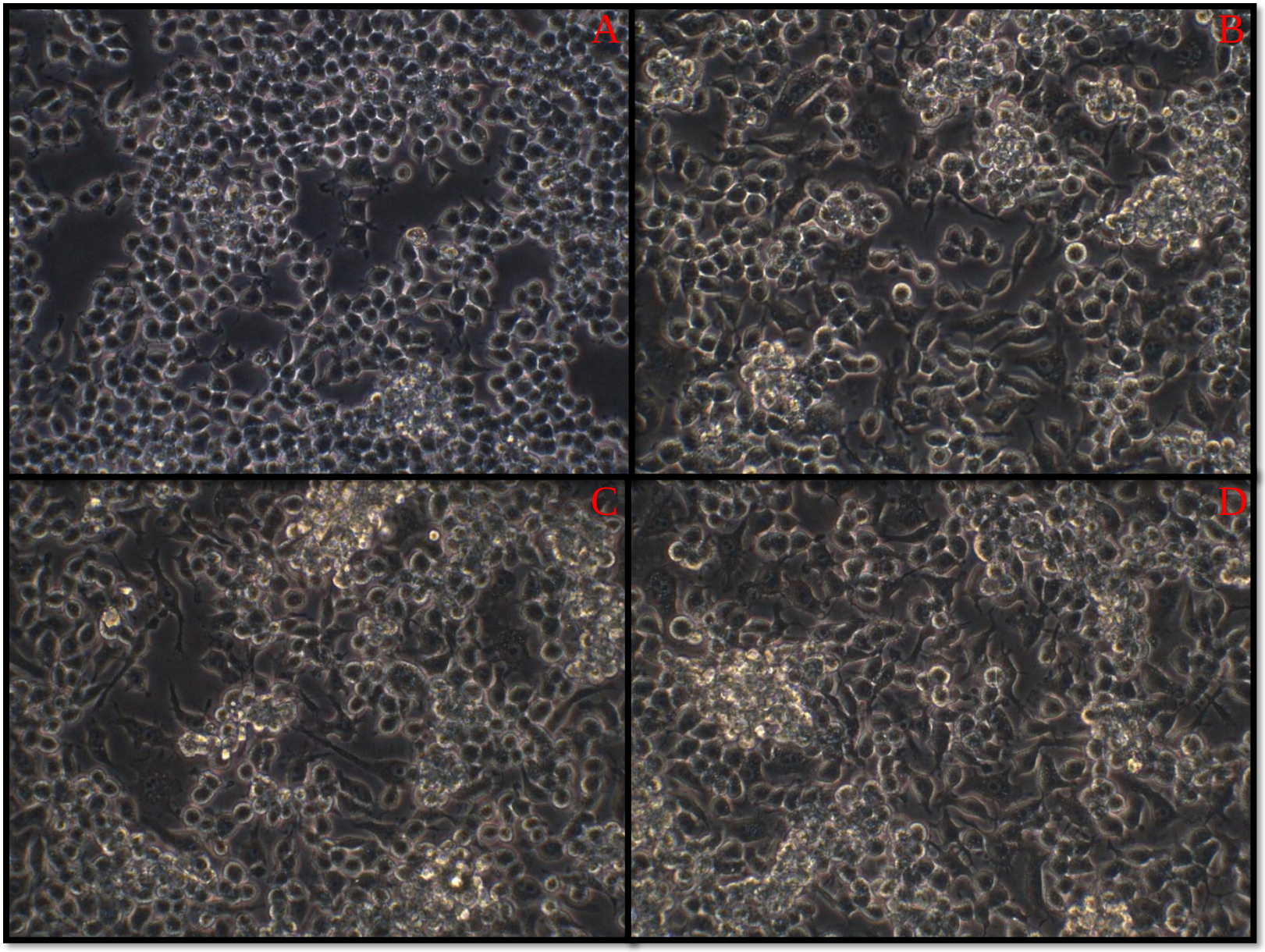

Figure 9. Photographs of RAW 264.7 macrophages with control and treatment. ***A:*** RAW 264.7 cells with 0 ng/mL LPS. ***B:*** RAW 264.7 cells with 0 ng/mL LPS and 0.375% GLY:EP. ***C:*** RAW 264.7 cells with 0 ng/mL LPS and 0.5% GLY:EP. ***D:*** RAW 264.7 cells with 0 ng/mL LPS and 0.75% GLY:EP. Images captured at 400x magnification using an Olympus B202 inverted microscope and OPELCO (optical elements corporation) software.

Discussion

I. Consistencies

The purpose of this project is to evaluate the immunomodulatory effects of GLY:EP stem and leaf extract on RAW 264.7 macrophages. Considering previous work with root extract showing anti-inflammatory effects (Nguyen 2022), as well as known constituents of EP having a potent anti-inflammatory effect on macrophages, we expected GLY:EP stem and leaf extract to have similar anti-inflammatory effects.

Our data suggests that EP has significant anti-inflammatory capacities when extracted in glycerol (figures 5 and 7). However, it is important to note that the glycerol solvent also displayed an independent effect in reducing NO in supernatant, suggesting a synergistic effect. Our data also suggests a potential anti-inflammatory capacity when extracted in ethanol (figures 2, 3, and 4). A study by Zhai et al. suggests chicoric acid is largely extracted from EtOH:EP extracts, yielding a significant anti-inflammatory effect (2007). Due to the range of active chemical constituents in EP, the variance in results from the EtOH experiments may be influenced by components that are both pro/anti-inflammatory in addition to independent effects of the EtOH solvent. Additionally, we found glycerol EP without LPS exhibited no significant difference in nitrites measured in supernatant when compared to the negative control. This finding is consistent with observations from Catanzaro et al. on enhancement of cytokine production with extract alone (2018).

Figure 8 shows there is also an appearance of comparatively higher numbers of larger macrophages from GLY:EP 1.5% extract compared to GLY control 1.5%. Figure 9 shows activity of the macrophages despite not being stimulated with LPS. Some of these macrophages

contain visible granules, which may be the polysaccharides (arabinogalactans) present in the EP aiding in activating the macrophages as described by Catanzaro et al. (2018).

II. Directions and Questions

Cytokine Analysis. TNF-alpha ELISA assay is a potential avenue to assess the cytokine modulation on the RAW 264.7 macrophages after treatment. TNF-alpha is a pro-inflammatory cytokine. Given the results of our experiments, a lower quantity of TNF-alpha in supernatant would be expected as was suggested by Raduner et al. (2006). Additionally, increased presence of anti-inflammatory cytokine interleukin-10 would be expected when compared to the controls. An increase in IL-10 production and respective decrease in TNF-alpha expression would be consistent with the findings of Chicca et al. and suggest a more comprehensive anti-inflammatory modulation due to EP (2009). In general, other cytokines must be evaluated to provide a bigger picture on the complexities of EP constituents and how they modulate inflammatory response together.

Macrophage Phenotype. Evaluation of M1/M2 phenotype after treatment, too, may be an important future direction. Alternatively activated M2 macrophages are known for their anti-inflammatory nature (Baseler et al. 2016; David et al. 2015). The anti-inflammatory cytokine IL-10 is released by CD4 positive T-cells as well as M2 macrophages (Baseler et al. 2016; David et al. 2015). IL-10 can limit NO production when signaling to M1 macrophages (Baseler et al. 2016). M2 macrophages can be polarized by IL-10 signaling (Lopes et al. 2016). The use of LPS in our treatments preferentially polarize the RAW 264.7 cells into the M1 phenotype. M1 macrophages do not typically produce IL-10, but Raduner et al. mentions that CB_2 binding through isobutylamides increases production of IL-10 cytokines in LPS stimulated macrophages

(2006). Which leads to the question of; does the IL-10 upregulated from CB_2 binding cause a phenotypic shift in a portion of the macrophage population that further potentiates the anti-inflammatory effects seen in EP?

NO Release. While death or activation state of the macrophages can influence nitrites measured in supernatant, one question that was brought up was the potential for retention of nitrites inside of the cell, rather than release of NO out into the environment. The appearances of the macrophages in figure 8 informed this question, as they are clearly activated. Larger granules are clearly present in the cytoplasm, but literature discussing possible retention of nitrites in the cell was not found upon search.

III. Limited Results

Due to the specificity of the Griess assay, the only thing we can be sure of when considering the results shown by spectrophotometry is that there may or may not be a reduction of NO in the supernatant. The assay does not evaluate why the phenomenon measured occurs, but that it occurs. Potential explanations of influence on what is observed in the referenced figures include; cell-death, activation state of cell, or concentrations of active components in the extracts.

Cell Death. If the solvent or treatment is killing the cells before they produce an amount of NO expected for the concentration of stimulatory agent, a reduction of NO in supernatant is not implausible. We suspect part of the reason why the EtOH:EP extracts were so variable was because the solvent could have been compromising the cells and causing them to lyse. Though, at which point in the treatment time this occurs is not clear. Figure 3 shows a clear significant

difference on the 1.5% and 3% EtOH controls when compared to the positive control, which may mean that the high EtOH concentration causes the cells to die before all cells have undergone inflammatory pathways. Though, the extract could have been working as intended and inhibited the inflammatory pathways of the macrophages. However, figure 2 shows a highly variable quantity of nitrites even at lower concentrations of EtOH, which further shrouds any potential effects that may be present. Pictures were not taken at this stage to assess the cells visually and a cell viability assay was not conducted to answer this question.

For the glycerol extracts, figures 5 and 7 show a consistent dose dependent effect for both the GLY:EP extracts and the glycerol control. The same potential can be applied to the glycerol extracts as ethanol, meaning that the treatments could be killing the cells. Pictures taken at this stage show that the cells are alive and activated when treated with GLY:EP extract and glycerol control (figure 8). This observation suggests that the anti-inflammatory effects seen in figures 5 and 7 are due to the chemical constituents of the extract, rather than the solvent compromising the cells. Though, no cell viability assay was conducted to answer this question without doubts. It is important to note, however, that the data suggests the glycerol control to have a significant independent effect on the cells at 1.5% and 3% concentrations. Therefore, effects of the EP extract may work synergistically with this solvent.

Concentrations of Active Components in Extract. The methods section of this study details glycerol EP extraction via a two-step process. This process generated a working extract after centrifuging an initial crude extract. Two working extracts were used in the glycerol EP treatments; working extract 1 that yielded the results in figures 5 and 7, and working extract 2 that resulted in figure 6. In retrospect, it may be possible that the working extracts have a different chemical profile due to plant matter presence and viscosity of the crude extract

influencing the vortex procedure. Figures 5 and 7 show consistent results, with a similar dose dependent pattern inhibition of nitrites in supernatant. However, figure 6 shows no significant differences due to the high standard variance of each treatment. The glycerol control of figure 6 has low standard variance, but also features unexpected nitrite levels in supernatant, so it is also possible the cells were not behaving typically on that day. The high variance of the GLY:EP + LPS treatment groups still remains, though, which may be in part due to a different concentration of chemicals present in the working extracts. Due to technical limitations, actual measurement of extract constituents and concentrations is not possible for the scope of this project. However, Zhai et al. have noted that EtOH:EP extracts contain the anti-inflammatory caffeic acid derivate of chicoric acid (2007). Ciganović et al. have noted that GLY:EP extracts also contain the anti-inflammatory caffeic acid derivatives of chicoric acid as well as caftaric acid (2023).

Lack of Standardization. One of the most important limitations to discuss is lack of standardization for *Echinacea* preparations. Currently, many market preparations are loosely regulated without FDA approval and contain a proprietary blend in their ingredients. There are also other products such as tinctures, EP succus, and fresh flower (EP) juice (Catanzaro et al. 2018) that all contain different chemical concentration profiles, and therefore different modulatory effects, due to their extraction methods (Bałan, et al. 2016). A study by David Senchina et al. utilizes varying extracts of *Echinacea* species root and effects on human peripheral blood mononuclear cells (2005). Cytokine assays were significantly influenced depending on extraction solvent, e.g., cold water, hot water, ethanol, and duration of extraction, e.g., 1 day and 4 days (Senchina et al. 2005). The upbringing of the EP plants harvested also influences chemical concentrations within the root and above ground parts. To alleviate

inconsistencies in research, great care must be taken to standardize procedures of harvest and extraction, as well as standardizing dose concentrations of the active components in EP.

Reduced System. Something important to recognize is the reduced system model we are working with. Macrophages are one of several white blood cells within the innate immune system. The potent anti-inflammatory effects suggested in this study among others referenced may in part be due to the effects of EP purely on macrophages alone. When introduced in complete living systems, perhaps immunostimulatory effects of EP will be present and measurable with the full context of the innate immune system to interact with the extract.

IV. Conclusion

The initial expectations for this project was for EP to modulate a reduced NO production in RAW 264.7 macrophages, measured through nitrites detected in supernatant. The data suggests that GLY:EP extracts have a statistically significant dose dependent decrease in supernatant nitrites when compared to the positive control of 100 ng/mL of LPS. For EtOH:EP extracts, there is a potential significant dose dependent decrease in supernatant nitrites, however two of the three experiments yielded inconclusive results. For the GLY:EP treatment groups without LPS, the data suggests no statistical differences in supernatant nitrites compared to the negative control of 0 ng/mL of LPS. Pictures taken at 400x magnification do show slight activation among the treatment groups, whereas the negative control features no activation.

Conclusions for EP's immunomodulatory effects as a whole cannot be drawn, as nitrites were the only measure of this question. Until further research is conducted on the influence of EP extracts on cytokine production, this study is only able to argue for an anti-inflammatory effect only within the context of NO production upon stimulation.

References

Bałan BJ, Sokolnicka I, Skopińska-Różewska E, Skopiński P. 2016. The modulatory influence of some Echinacea-based remedies on antibody production and cellular immunity in mice. Cent Eur J Immunol. 41(1):12-8. DOI: 10.5114/ceji.2016.58813.

Barrett B. 2003. Medicinal properties of *Echinacea*: A critical review. Phytomedicine. 10(1):66-86. DOI: 10.1078/094471103321648692.

Baseler WA, Davies LC, Quigley L, Ridnour LA, Weiss JM, Hussain SP, et al. 2016. Autocrine IL-10 functions as a rheostat for M1 macrophage glycolytic commitment by tuning nitric oxide production. Redox Biol. 10:12-23. DOI: 10.1016/j.redox.2016.09.005.

Bauer R. 1996. Echinacea-Drogen: Wirkungen und Wirksubstanzen [Echinacea-containing drugs: Effects and active constituents. Translated by Beate V. Foit, Santa Cruz. CA.]. Z Arztl Fortbild (Jena). 90(2):111-115. PMID: 8686333.

Bauer R, Jurcic K, Puhlmann J, Wagner H. 1988. Immunologische In-vivo und In-vitro-Untersuchungen mit Echinacea-Extrakten. (Immunological in vivo and in vitro examinations of Echinacea extracts.) Arzneimittelforschung. 38(2):276-281. (In German) PMID: 3370076.

Borchers AT, Keen CL, Stern JS, Gershwin ME. 2000. Inflammation and Native American medicine: the role of botanicals. Am J Clin Nutr. 72(2):339-347. DOI: 10.1093/ajcn/72.2.339.

Bryan NS, Grisham MB. 2007. Methods to detect nitric oxide and its metabolites in biological samples. Free Radic Biol Med. 43(5):645-57. DOI: 10.1016/j.freeradbiomed.2007.04.026.

Catanzaro M, Corsini E, Rosini M, Racchi M, and Lanni C. 2018. Immunomodulators inspired by nature; a review on Curcumin and Echinacea. Molecules. 23(11):2778. DOI: 10.3990/molecules23112778.

Chicca A, Raduner S, Pellati F, Strompen T, Altmann KH, Schoop R, et al. 2009. Synergistic immunomopharmacological effects of N-alkylamides in Echinacea purpurea herbal extracts. Int Immunopharmacol. 9(7-8):850-858. DOI: 10.1016/j.intimp.2009.03.006.

Ciganović P, Jakupović L, Momchev P, Nižić Nodilo L, Hafner A, Zovko Končić M. 2023. Extraction Optimization, Antioxidant, Cosmeceutical and Wound Healing Potential of Echinacea purpurea Glycerolic Extracts. Molecules. 28(3):1177. DOI: 10.3390/molecules28031177.

Classen B, Thude S, Blaschek W, Wack M, Bodinet C. 2006. Immunomodulatory effects of arabinogalactan-proteins from Baptisia and Echinacea. Phytomedicine. 13(9-10):688-694. DOI: 10.1016/j.phymed.2005.10.004.

CNX OpenStax. 2016. OpenStax Microbiology. Figure 3.28: Lipopolysaccharide. ISBN-13: 978-1-947172-23-4. Licensed under Creative Commons Attribution License v4.0. [Accessed on: 11 May, 2023]. Link.

Coligan JE, Kruisbeek AM, Margulies DH, Shevach EM, Strober W. 1991. Current Protocols in Immunology. Vol. 3, In: *Immunology—Laboratory Manuals.* ISBN: 0-471-52276-7.

Collins E, Berkoff N. 1999. Everything You Need to Know About Echinacea and Immunity. *The Natural Pharmacist.* Prima Publishing. ISBN: 0-7615-1558-5.

Farhana A, Khan YS. 2023. Biochemistry, Lipopolysaccharide. [Updated 2022 Apr 21]. In: StatPearls [Internet]. Treasure Island (FL): StatPearls Publishing. Available from: https://www.ncbi.nlm.nih.gov/books/NBK554414/.

Green SJ, Nacy CA. 1993. Antimicrobial and immunopathogenic effects of cytokine-induced nitric oxide synthesis. Curr. Opin. Infect. Dis. 6:384-396. ISSN: 0951-7375.

Lopes RL, Borges TJ, Zanin RF, Bonorino C. 2016. IL-10 is required for polarization of macrophages to M2-like phenotype by mycobacterial DnaK (heat shock protein 70). J Cytol. 85:123-129. DOI: 10.1016/j.cyto.2016.06.018.

Luettig B, Steinmüller C, Gifford GE, Wagner H, Lohmann-Matthes M-L. 1989. Macrophage activation by the polysaccharide arabinogalactan isolated from plant cell cultures of *Echinacea purpurea*. Jour Nation Can Inst. 81(9):669-675. DOI: 10.1093/jnci/81.9.669.

Manayi A, Vazirian M, Saeidnia S. 2015. *Echinacea purpurea*: Pharmacology, phytochemistry, and analysis methods. Pharmacogn Rev. 9(17):63-72. DOI: 10.4103/0973-7847.156353.

Matthias A, Banbury L, Bone KM, Leach DN, Lehmann RP. 2008. Echinacea alkylamides modulate induced immune responses in T-cells. Fitoterapia. 79(1):53-58. DOI: 10.1016/j.fitote.2007.07.012.

Meng F, Lowell CA. 1997. Lipopolysaccharide (LPS)-induced macrophage activation and signal transduction in the absence of Src-family kinases Hck, Fgr, and Lyn. J Exp Med. 185(9):1661-1670. DOI: 10.1084/jem.185.9.1661.

Nguyen TT. 2022. The Effect of Ethanolic Echinacea purpurea Root Extracts on the Production of Pro-Inflammatory Mediators in RAW 264.7 Murine Macrophages [thesis]. [Lynchburg (VA)]: University of Lynchburg.

Park B, Lee JO. 2013. Recognition of lipopolysaccharide pattern by TLR4 complexes. Exp Mol Med. 45(e66). DOI: 10.1038/emm.2013.97.

Promega. 2009. Griess Reagent System: Instructions for use of Product G2930. Technical Bulletin. [Accessed on: 2 May, 2023]. Link.

Raduner S, Majewska A, Chen JZ, Xie XQ, Hamon J, Faller B, et al. 2006. Alkylamides from *Echinacea* are a new class of cannabinomimetics: cannabinoid type 2 receptor-dependent and -independent immunomodulatory effects. Jour Biol Chem. 28(20):14192-14206. DOI: 10.1074/jbc.M601074200.

Raschke WC, Baird S, Ralph P, Nakoinz I. 1978. Functional Macrophage Cell Lines Transformed by Abelson Leukemia Virus. Cell. 15(1):261-267. DOI: 10.1016/0092-8674(78)90101-0.

Ren W, Ban J, Xia Y, Zhou F, Yuan C, Jia H, et al. 2023. Echinacea purpurea-derived homogeneous polysaccharide exerts anti-tumor efficacy via facilitating M1 macrophage polarization. Innovation (Camb). 4(2):100391. DOI: 10.1016/j.xinn.2023.100391.

Sanin DE, Prendergast CT, Mountford AP. 2015. IL-10 Production in Macrophages is Regulated by a TLR-Driven CREB-Mediated Mechanism That is Linked to Genes Involved in Cell Metabolism. J Immunol. 195(3):1218–1232. DOI: 10.4049/jimmunol.1500146.

Senchina DS, McCann DA, Asp JM, Johnson JA, Cunnick JE, Kaiser MS, et al. 2005. Changes in immunomodulatory properties of *Echinacea* spp. root infusions and tinctures stored at 4 °C for four days. Clin Chim Act. 335(1-2):67-82. DOI: 10.1016/j.cccn.2004.12.013.

Taciak B, Białasek M, Braniewska A, Sas Z, Sawicka P, Kiraga Ł, et al. 2018. Evaluation of phenotypic and functional stability of RAW 264.7 cell line through serial passages. PLoS One. 13(6):e0198943. DOI: 10.1371/journal.pone.0198943.

Yang G, Li K, Liu C, Peng P, Bai M, Sun J, et al. 2018. A comparison of the immunostimulatory effects of polysaccharides from tetraploid and diploid echinacea purpurea. Biomed Res Int. ID: 8628531. DOI: 10.1155/2018/8628531.

Zhai Z, Haney D, Wu L, Solco A, Murphy PA, Wurtele ES, et al. 2007. Alcohol extracts of Echinacea inhibit production of nitric oxide and tumor necrosis factor-alpha by macrophages in vitro. Food Agric Immunol. 18(3-4):221-236. DOI: 10.1080/09540100701797363.

Appendix A

Statistical Analyses of Experimental Data

Table A1. Oct. 1, 2022 EtOH Exp. 1 One-Way ANOVA with 0 ng/mL LPS as control.

ANOVA

Nitrite

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	21765.114	7	3109.302	33.694	<.001
Within Groups	2214.721	24	92.280		
Total	23979.834	31			

Table A2. Oct. 1, 2022 EtOH Exp. 1 Dunnett's 2-Sided T-Test with 0 ng/mL LPS as control.

Multiple Comparisons

Dependent Variable: Nitrite

Dunnett t (2-sided)[a]

(I) Category	(J) Category	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	8	77.0370370*	6.792643857	<.001	57.92101449	96.15305958
2	8	78.0092593*	6.792643857	<.001	58.89323671	97.12528180
3	8	80.5555556*	6.792643857	<.001	61.43953301	99.67157810
4	8	74.7685185*	6.792643857	<.001	55.65249597	93.88454106
5	8	84.7685185*	6.792643857	<.001	65.65249597	103.8845411
6	8	80.2777778*	6.792643857	<.001	61.16175523	99.39380032
7	8	68.2407407*	6.792643857	<.001	49.12471820	87.35676329

*. The mean difference is significant at the 0.05 level.

a. Dunnett t-tests treat one group as a control, and compare all other groups against it.

Table A3. Oct. 1, 2022 EtOH Exp. 1 One-Way ANOVA with 100 ng/mL LPS as control.

ANOVA

Nitrite

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	21765.114	7	3109.302	33.694	<.001
Within Groups	2214.721	24	92.280		
Total	23979.834	31			

Table A4. Oct. 1, 2022 EtOH Exp. 1 Dunnett's 2-Sided T-Test with 100 ng/mL LPS as control.

Multiple Comparisons

Dependent Variable: Nitrite

Dunnett t (2-sided)[a]

(I) Category	(J) Category	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	8	-77.0370370*	6.792643857	<.001	-96.1530596	-57.9210145
2	8	.9722222222	6.792643857	1.000	-18.1438003	20.08824477
3	8	3.518518519	6.792643857	.994	-15.5975040	22.63454106
4	8	-2.26851852	6.792643857	1.000	-21.3845411	16.84750403
5	8	7.731481481	6.792643857	.770	-11.3845411	26.84750403
6	8	3.240740741	6.792643857	.996	-15.8752818	22.35676329
7	8	-8.79629630	6.792643857	.664	-27.9123188	10.31972625

*. The mean difference is significant at the 0.05 level.

a. Dunnett t-tests treat one group as a control, and compare all other groups against it.

Table A5. Oct. 21, 2022 EtOH Exp. 2 One-Way ANOVA with 0 ng/mL LPS as control.

ANOVA

Nitrite

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	23133.721	9	2570.413	77.358	<.001
Within Groups	963.600	29	33.228		
Total	24097.321	38			

Table A6. Oct. 21, 2022 EtOH Exp. 2 Dunnett's 2-Sided T-Test with 0 ng/mL LPS as control.

Multiple Comparisons

Dependent Variable: Nitrite

Dunnett t (2-sided)[a]

(I) Category	(J) Category	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	10	66.0169492*	4.076002139	<.001	54.33773819	77.69616011
2	10	49.0677966*	4.076002139	<.001	37.38858565	60.74700757
3	10	47.3305085*	4.076002139	<.001	35.65129751	59.00971944
4	10	15.5508475*	4.076002139	.005	3.871636496	27.23005842
5	10	-.423728814	4.076002139	1.000	-12.1029398	11.25548215
6	10	65.8757062*	4.402585491	<.001	53.26071658	78.49069585
7	10	65.0000000*	4.076002139	<.001	53.32078904	76.67921096
8	10	40.1694915*	4.076002139	<.001	28.49028056	51.84870249
9	10	30.6355932*	4.076002139	<.001	18.95638226	42.31480418

*. The mean difference is significant at the 0.05 level.

a. Dunnett t-tests treat one group as a control, and compare all other groups against it.

Table A7. Oct. 21, 2022 EtOH Exp. 2 One-Way ANOVA with 100 ng/mL LPS as control.

ANOVA

Nitrite

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	23133.721	9	2570.413	77.358	<.001
Within Groups	963.600	29	33.228		
Total	24097.321	38			

Table A8. Oct. 21, 2022 EtOH Exp. 2 Dunnett's 2-Sided T-Test with 100 ng/mL LPS as control.

Multiple Comparisons

Dependent Variable: Nitrite

Dunnett t (2-sided)[a]

(I) Category	(J) Category	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	10	-66.0169492*	4.076002139	<.001	-77.6961601	-54.3377382
2	10	-16.9491525*	4.076002139	.002	-28.6283635	-5.26994158
3	10	-18.6864407*	4.076002139	<.001	-30.3656516	-7.00722972
4	10	-50.4661017*	4.076002139	<.001	-62.1453127	-38.7868907
5	10	-66.4406780*	4.076002139	<.001	-78.1198889	-54.7614670
6	10	-.141242938	4.402585491	1.000	-12.7562326	12.47374670
7	10	-1.01694915	4.076002139	1.000	-12.6961601	10.66226181
8	10	-25.8474576*	4.076002139	<.001	-37.5266686	-14.1682467
9	10	-35.3813559*	4.076002139	<.001	-47.0605669	-23.7021450

*. The mean difference is significant at the 0.05 level.

a. Dunnett t-tests treat one group as a control, and compare all other groups against it.

Table A9. Feb. 7, 2023 EtOH Exp. 3 One-Way ANOVA with 0 ng/mL LPS as control.

ANOVA

Nitrite

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	5746.213	7	820.888	4.137	.004
Within Groups	4762.098	24	198.421		
Total	10508.312	31			

Table A10. Feb. 7, 2023 EtOH Exp. 3 Dunnett's 2-Sided T-Test with 0 ng/mL LPS as control.

Multiple Comparisons

Dependent Variable: Nitrite

Dunnett t (2-sided)[a]

(I) Category	(J) Category	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	8	33.1521739*	9.960440815	.016	5.121260595	61.18308723
2	8	42.8804348*	9.960440815	.001	14.84952146	70.91134810
3	8	35.0543478*	9.960440815	.010	7.023434508	63.08526114
4	8	35.2173913*	9.960440815	.010	7.186477986	63.24830462
5	8	45.5978261*	9.960440815	<.001	17.56691277	73.62873941
6	8	30.0543478*	9.960440815	.032	2.023434508	58.08526114
7	8	21.84782609	9.960440815	.174	-6.18308723	49.87873941

*. The mean difference is significant at the 0.05 level.

a. Dunnett t-tests treat one group as a control, and compare all other groups against it.

Table A11. Feb. 7, 2023 EtOH Exp. 3 One-Way ANOVA with 100 ng/mL LPS as control.

ANOVA

Nitrite

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	5746.213	7	820.888	4.137	.004
Within Groups	4762.098	24	198.421		
Total	10508.312	31			

Table A12. Feb. 7, 2023 EtOH Exp. 3 Dunnett's 2-Sided T-Test with 100 ng/mL LPS as control.

Multiple Comparisons

Dependent Variable: Nitrite

Dunnett t (2-sided)[a]

(I) Category	(J) Category	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	8	-33.1521739*	9.960440815	.016	-61.1830872	-5.12126059
2	8	9.728260870	9.960440815	.866	-18.3026524	37.75917419
3	8	1.902173913	9.960440815	1.000	-26.1287394	29.93308723
4	8	2.065217391	9.960440815	1.000	-25.9656959	30.09613071
5	8	12.44565217	9.960440815	.695	-15.5852611	40.47656549
6	8	-3.09782609	9.960440815	1.000	-31.1287394	24.93308723
7	8	-11.3043478	9.960440815	.772	-39.3352611	16.72656549

*. The mean difference is significant at the 0.05 level.

a. Dunnett t-tests treat one group as a control, and compare all other groups against it.

Table A13. Feb. 19, 2023 GLY Exp. 1 One-Way ANOVA with 0 ng/mL LPS as control.

ANOVA

Nitrite

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	9513.739	7	1359.106	374.366	<.001
Within Groups	87.130	24	3.630		
Total	9600.869	31			

Table A14. Feb. 19, 2023 GLY Exp. 1 Dunnett's 2-Sided T-Test with 0 ng/mL LPS as control.

Multiple Comparisons

Dependent Variable: Nitrite

Dunnett t (2-sided)[a]

(I) Category	(J) Category	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	8	44.3269231*	1.347298049	<.001	40.53532434	48.11852181
2	8	20.0480769*	1.347298049	<.001	16.25647819	23.83967566
3	8	11.0096154*	1.347298049	<.001	7.218016648	14.80121412
4	8	9.95192308*	1.347298049	<.001	6.160324340	13.74352181
5	8	48.5096154*	1.347298049	<.001	44.71801665	52.30121412
6	8	44.2307692*	1.347298049	<.001	40.43917049	48.02236797
7	8	21.7307692*	1.347298049	<.001	17.93917049	25.52236797

*. The mean difference is significant at the 0.05 level.

a. Dunnett t-tests treat one group as a control, and compare all other groups against it.

Table A15. Feb. 19, 2023 GLY Exp. 1 One-Way ANOVA with 100 ng/mL LPS as control

ANOVA

Nitrite

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	9513.739	7	1359.106	374.366	<.001
Within Groups	87.130	24	3.630		
Total	9600.869	31			

Table A16. Feb. 19, 2023 GLY Exp. 1 Dunnett's 2-Sided T-Test with 100 ng/mL LPS as control.

Multiple Comparisons

Dependent Variable: Nitrite

Dunnett t (2-sided)[a]

(I) Category	(J) Category	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	8	-44.3269231*	1.347298049	<.001	-48.1185218	-40.5353243
2	8	-24.2788462*	1.347298049	<.001	-28.0704449	-20.4872474
3	8	-33.3173077*	1.347298049	<.001	-37.1089064	-29.5257090
4	8	-34.3750000*	1.347298049	<.001	-38.1665987	-30.5834013
5	8	4.18269231*	1.347298049	.026	.3910935708	7.974291045
6	8	-.096153846	1.347298049	1.000	-3.88775258	3.695444891
7	8	-22.5961538*	1.347298049	<.001	-26.3877526	-18.8045551

*. The mean difference is significant at the 0.05 level.

a. Dunnett t-tests treat one group as a control, and compare all other groups against it.

Table A17. Feb. 27, 2023 GLY Exp. 2 One-Way ANOVA with 0 ng/mL LPS as control.

ANOVA

Nitrite

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	8649.524	10	864.952	29.947	<.001
Within Groups	953.121	33	28.882		
Total	9602.646	43			

Table A18. Feb. 27, 2023 GLY Exp. 2 Dunnett's 2-Sided T-Test with 0 ng/mL LPS as control.

Multiple Comparisons

Dependent Variable: Nitrite

Dunnett t (2-sided)[a]

(I) Category	(J) Category	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	11	29.7413793*	3.800162046	<.001	18.82172628	40.66103234
2	11	32.2413793*	3.800162046	<.001	21.32172628	43.16103234
3	11	33.7931034*	3.800162046	<.001	22.87345042	44.71275647
4	11	36.4655172*	3.800162046	<.001	25.54586421	47.38517027
5	11	.7327586207	3.800162046	1.000	-10.1868944	11.65241165
6	11	1.250000000	3.800162046	1.000	-9.66965303	12.16965303
7	11	1.810344828	3.800162046	1.000	-9.10930820	12.72999785
8	11	23.0172414*	3.800162046	<.001	12.09758835	33.93689441
9	11	21.7241379*	3.800162046	<.001	10.80448490	32.64379096
10	11	23.5775862*	3.800162046	<.001	12.65793318	34.49723923

*. The mean difference is significant at the 0.05 level.

a. Dunnett t-tests treat one group as a control, and compare all other groups against it.

Table A19. Feb. 27, 2023 GLY Exp. 2 One-Way ANOVA with 100 ng/mL LPS as control.

ANOVA

Nitrite

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	8649.524	10	864.952	29.947	<.001
Within Groups	953.121	33	28.882		
Total	9602.646	43			

Table A20. Feb. 27, 2023 GLY Exp. 2 Dunnett's 2-Sided T-Test with 100 ng/mL LPS as control.

Multiple Comparisons

Dependent Variable: Nitrite

Dunnett t (2-sided)[a]

(I) Category	(J) Category	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	11	-29.7413793*	3.800162046	<.001	-40.6610323	-18.8217263
2	11	2.500000000	3.800162046	.994	-8.41965303	13.41965303
3	11	4.051724138	3.800162046	.890	-6.86792889	14.97137716
4	11	6.724137931	3.800162046	.423	-4.19551510	17.64379096
5	11	-29.0086207*	3.800162046	<.001	-39.9282737	-18.0889677
6	11	-28.4913793*	3.800162046	<.001	-39.4110323	-17.5717263
7	11	-27.9310345*	3.800162046	<.001	-38.8506875	-17.0113815
8	11	-6.72413793	3.800162046	.423	-17.6437910	4.195515095
9	11	-8.01724138	3.800162046	.242	-18.9368944	2.902411647
10	11	-6.16379310	3.800162046	.521	-17.0834461	4.755859923

*. The mean difference is significant at the 0.05 level.

a. Dunnett t-tests treat one group as a control, and compare all other groups against it.

Table A21. Mar. 3, 2023 GLY Exp. 3 One-Way ANOVA with 0 ng/mL LPS as control.

ANOVA

Nitrite

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	37627.198	9	4180.800	1214.910	<.001
Within Groups	103.237	30	3.441		
Total	37730.435	39			

Table A22. Mar. 3, 2023 GLY Exp. 3 Dunnett's 2-Sided T-Test with 0 ng/mL LPS as control.

Multiple Comparisons

Dependent Variable: Nitrite

Dunnett t (2-sided)[a]

(I) Category	(J) Category	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	10	80.1086957*	1.311724311	<.001	76.36297086	83.85442044
2	10	3.641304348	1.311724311	.060	-.104420442	7.387029138
3	10	1.739130435	1.311724311	.706	-2.00659436	5.484855225
4	10	3.152173913	1.311724311	.131	-.593550877	6.897898703
5	10	66.5760870*	1.311724311	<.001	62.83036217	70.32181175
6	10	51.8478261*	1.311724311	<.001	48.10210130	55.59355088
7	10	27.3913043*	1.311724311	<.001	23.64557956	31.13702914
8	10	70.3804348*	1.311724311	<.001	66.63470999	74.12615957
9	10	56.5760870*	1.311724311	<.001	52.83036217	60.32181175

*. The mean difference is significant at the 0.05 level.

a. Dunnett t-tests treat one group as a control, and compare all other groups against it.

Table A23. Mar. 3, 2023 GLY Exp. 3 One-Way ANOVA with 100 ng/mL LPS as control.

ANOVA

Nitrite

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	37627.198	9	4180.800	1214.910	<.001
Within Groups	103.237	30	3.441		
Total	37730.435	39			

Table A24. Mar. 3, 2023 GLY Exp. 3 Dunnett's 2-Sided T-Test with 100 ng/mL LPS as control.

Multiple Comparisons

Dependent Variable: Nitrite

Dunnett t (2-sided)[a]

(I) Category	(J) Category	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
1	10	-80.1086957*	1.311724311	<.001	-83.8544204	-76.3629709
2	10	-76.4673913*	1.311724311	<.001	-80.2131161	-72.7216665
3	10	-78.3695652*	1.311724311	<.001	-82.1152900	-74.6238404
4	10	-76.9565217*	1.311724311	<.001	-80.7022465	-73.2107969
5	10	-13.5326087*	1.311724311	<.001	-17.2783335	-9.78688391
6	10	-28.2608696*	1.311724311	<.001	-32.0065944	-24.5151448
7	10	-52.7173913*	1.311724311	<.001	-56.4631161	-48.9716665
8	10	-9.72826087*	1.311724311	<.001	-13.4739857	-5.98253608
9	10	-23.5326087*	1.311724311	<.001	-27.2783335	-19.7868839

*. The mean difference is significant at the 0.05 level.

a. Dunnett t-tests treat one group as a control, and compare all other groups against it.

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