Isolation and Characterization of an Immunomodulatory Compound from a Plant Used in Traditional Chinese Medicine

By

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I dedicate this research to Franky.

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I would like to thank my wife, Meredith, and our children, Lucy and Annabelle, for their encouragement and patience. I would like to thank my committee members for their time, advice, support, and conversation.

ABSTRACT

Inflammation is a normal part of a healthy immune response. However, when inflammatory pathways are overactive or inappropriately activated, diseases such as atopic dermatitis, Crohn's disease, and asthma can occur. Current anti-inflammatory drugs have many side effects and can be very expensive. The purpose of this study was to isolate and characterize an immunomodulatory compound in *Mangifera persiciformis,* a plant used in traditional Chinese medicine.

A bioassay-guided fractionation of *M. persiciformis* was conducted. After fractionating extract 23B of M. *persiciformis* using flash chromatography, enzyme-linked immunosorbent assays (ELISAs) and cell viability assays were used to identify fractions with nontoxic, immunomodulatory properties in THP-1 human monocyte-derived macrophages. Fractions 4, 5, and 6 of 23B caused a reduced cytokine response to bacterial lipopolysaccharides (LPS). Only Fraction 4 maintained cell viability and was further fractionated until a single compound referred to as F4-2b was isolated. An attempt was made to characterize and identify this compound using NMR spectroscopy. The NMR spectroscopy revealed some structural characteristics of the F4-2b, but further isolation and purification will be required in order to identify the compound.

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INTRODUCTION

Inflammation is a normal part of a healthy immune response. Classically recognized as redness, swelling, pain, and warmth, it is the result of many cell signaling pathways. It is part of the body's response to pathogens or cellular damage, and a wide range of related and connected pathways can be activated, depending on the specific stimulus of the inflammation (Chen, et al. 2017). However, when inflammatory pathways are overactive or inappropriately activated, diseases can occur. Atopic dermatitis, Crohn's disease, asthma, and rheumatoid arthritis are just a few of the many diseases caused by or associated with dysfunctional or inappropriately activated inflammation pathways (Kuenzig, et al. 2018).

The communication between cells during inflammation is facilitated by cytokines. Cytokines are peptides secreted by cells and used for cell signaling. The signal is initiated when a cytokine binds to its respective receptor on the outside of a cell. Cytokines can have multiple receptors on different cell types, activating various processes depending on the context of the signaling. The binding of a cytokine to its receptor can start a cascade of reactions in the cell. Cytokines such as tumor necrosis factor alpha (TNF-α) can simultaneously activate cytokine pathways involving white blood cell recruitment, cell adhesion, vascular functions, apoptosis, cytokine production, and others. When kinase pathways such as the mitogen activated protein kinase (MAPK) (Figure 1) and Janus kinase (JAK) (Figure 2) are activated by TNF-α, their targets of phosphorylation facilitate proteolysis and the activation of transcription factors. TNF-α also activates pathways such

as the nuclear factor kB (NF-kB) pathway, which carries out the modulation of numerous cytokines and cell surface receptors.

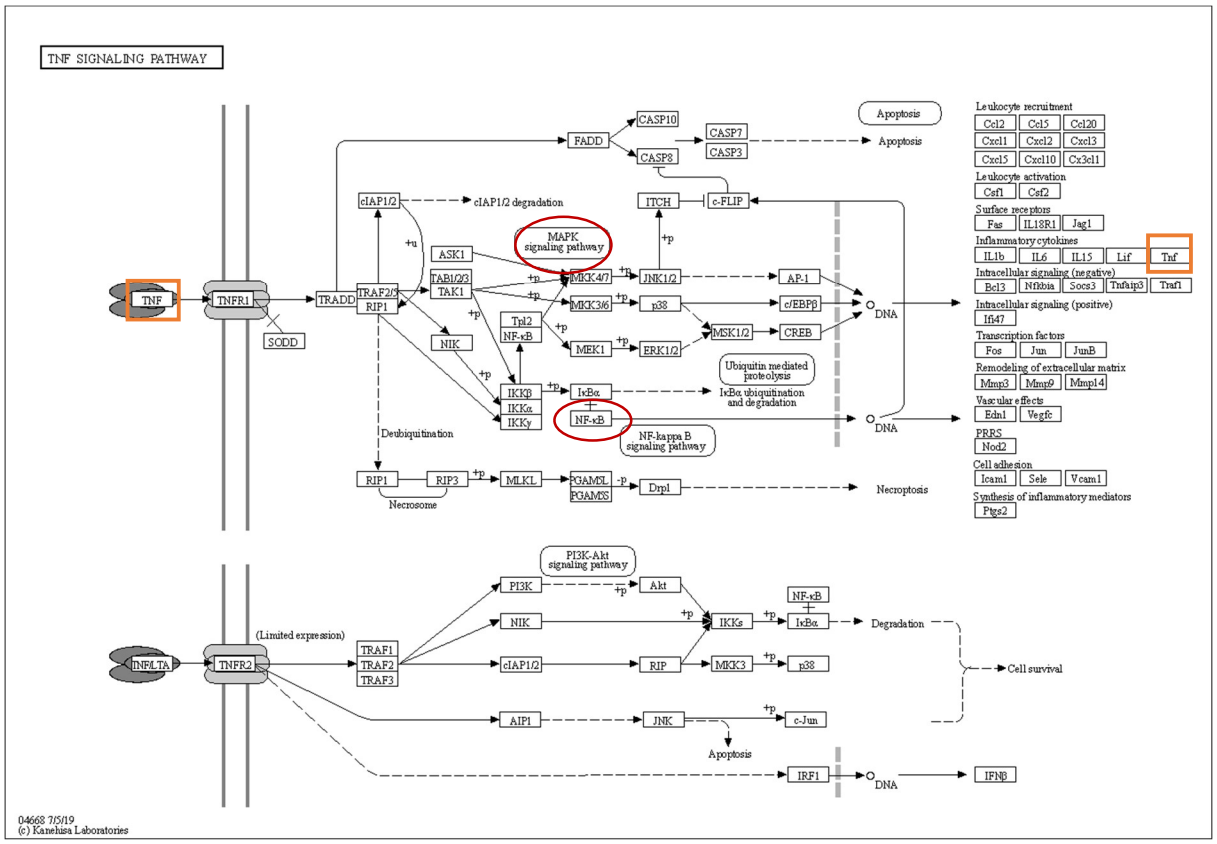

Figure 1. TNF Signaling Pathway

TNF-α activates numerous pathways, including the MAPK and NF-kB. This activation by TNF-α generates positive feedback, leading to the production of more TNF-α. In this figure, the MAPK and NF-kB inflammatory pathways are activated when the cytokine TNF binds to one of its receptors, TNFR1. (Kanehisa & Goto 2000).

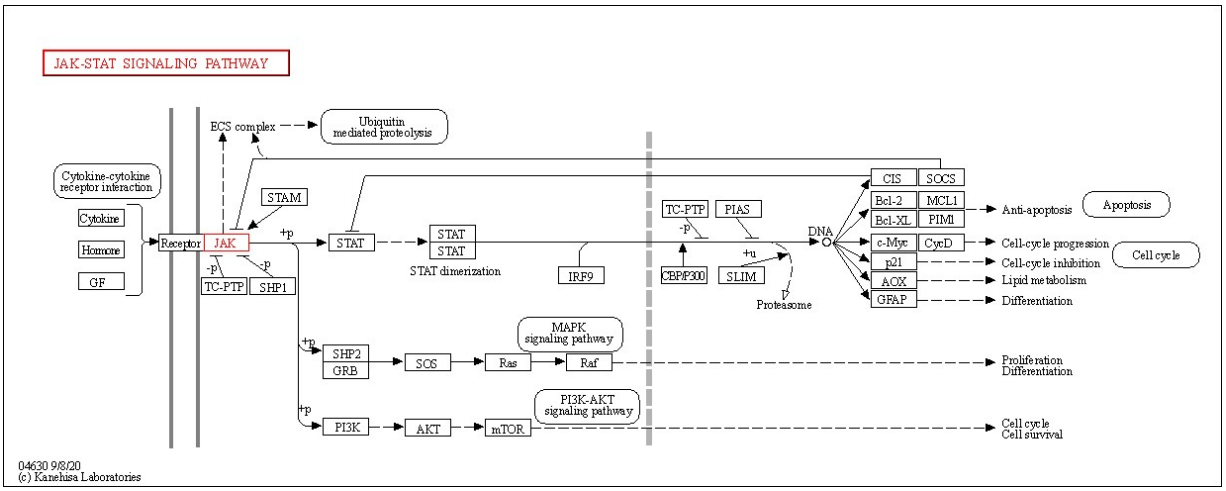

Figure 2. JAK-STAT Signaling Pathway

Multiple signaling pathways, including a JAK-STAT pathway, are initiated by the binding of a cytokine to its receptor on the cell surface. (Kanehisa & Goto 2000).

The complexity and variety of the numerous inflammatory signaling pathways presents many potential molecular targets for the inhibition or suppression of inflammation. This inhibition has the potential to be either broad-spectrum or specific, depending on the target and its respective role in the inflammatory process.

Current Options for Treating Inflammation

Corticosteroids, non-steroidal anti-inflammatory drugs (NSAIDs), and monoclonal antibody treatments are drugs commonly used to control inflammation. These drugs, however, are often associated with deleterious side effects. Corticosteroids suppress inflammation through both genomic and non-genomic actions (Alangari 2010). Corticosteroids first bind with receptor complexes in the cytosol before moving to the

nucleus to cause the downregulation or upregulation of genes by interacting with glucocorticoid-responsive elements (GREs), often located in promotor or enhancer regions of genes (Oakley & Cidlowski 2013). Desired targets of this action include the repression of genes coding for inflammatory cytokines such as TNF-α and interleukin 1-beta (IL-1β) as well as enzymes involved in inflammation such as the inducible nitric oxide synthase (iNOS) (Radomski, et al. 1990). Unfortunately, these are not the only GRE targets affected by corticosteroid complexes. Members of the collagen gene family and transforming growth factor (TGF) gene family are also suppressed, leading to side effects such as skin atrophy, osteoporosis, and disturbed wound healing (Wang, et al. 2018). Corticosteroids also inhibit the release of arachidonic acid, the precursor for prostaglandins and leukotrienes (Malcher-Lopes, et al. 2008). Although inhibiting the production of inflammatory prostaglandins produces the desired clinical effect of suppressing inflammation, there are constitutive functions of arachidonic acid derivatives such as platelet aggregation, renal function, and vasodilation (Wang, et al. 2021). Prolonged use of corticosteroids results in prolonged suppression of these homeostasis-related functions. NSAIDs share this problem. NSAIDs such as ibuprofen and naproxen block the cyclooxygenase (COX) enzymes (Moore, et al. 2015). These enzymes are responsible for converting arachidonic acid into prostaglandins. The products of constitutively active COX-1 are responsible for homeostasis functions, as well as gastroprotection from stomach acid. Inhibition of COX-1 leads to side effects associated with NSAIDs such as gastrointestinal damage and renal dysfunction. The use of NSAIDs with a higher affinity for inhibiting COX-2 than COX-1 support the validity of this proposed mechanism by having less of these specific side effects (Sostres, et al. 2013). However, even the COX-

2 selective inhibitors have serious side effects including liver damage and increased risk of cardiovascular disease.

More recently, monoclonal antibodies that prevent TNF-α from binding with its receptor have been synthesized and used to control inflammation. (Lis, et al. 2014). TNF-α is known to play a key role in inflammation, and inhibiting TNF activity has been shown to have a profound anti-inflammatory effect. These TNF blockers, as they are called, act in a more targeted manner than NSAIDs and corticosteroids. However, these biotherapeutics are very expensive and range in cost from approximately $18,000 to $28,000 per patient for a year of treatment (Bonafede, et al. 2014). Additionally, like other anti-inflammatory drugs, TNF blockers have many serious side effects. In the body, TNF-α exists in both a transmembrane form and a soluble form. These two forms have different functions. The soluble form perpetuates inflammatory pathways, but the transmembrane form plays a role in autoantigen tolerance and the innate immune response to infections (Faustman & Davis 2013, Vujanovic 2011). Inhibition of the transmembrane form of TNF-α is thought to be the cause of severe infections, the most serious side effect associated with TNF blockers (Lis, et al. 2014).

Traditional Medicine as a Source of New Drugs

Faced with the problems presented by current anti-inflammatory treatments, consumers are looking for affordable and safe alternatives. Natural products and natural product derivatives are a viable source for novel, effective drugs. Of the 1073 small molecule chemical entities approved by the FDA and similar organizations for therapeutic

use between 1981 and 2010, over 50% were either natural products, derivatives of natural products, or synthetically made natural product mimics (Newman and Cragg 2012). Over 10% of the World Health Organization's List of Essential Medicines is comprised of natural products and their derivatives (Veeresham 2015). Their prevalence in these two lists asserts that natural products are relevant as a source of therapeutic small molecules.

For thousands of years, trial and error on human subjects has identified an immense library of plants used in traditional medicine to treat a wide array of ailments and diseases. During the last few decades, drug developers have realized the potential in this library, and extensive effort has been put into the elucidation of the active components of these medicinal plants (Shakya 2016). The purpose of this project was to identify the active compound in a plant extract used in traditional Chinese medicine that has been shown to possess immunomodulatory properties. The extract used in this project was designated as 23B, and it is the ethyl acetate (EA) extract of *Mangifera persiciformis* branches and leaves.

Mangifera persiciformis

Teas and other preparations of *Mangifera indica*, a close relative of *M. persiciformis*, have been used medicinally for over 4,000 years (Shah 2010). A closely related species commonly known as mango, *M. indica*, has been shown to possess anti-inflammatory, anti-tumor, anti-viral, anti-fungal, and many other medically important properties. Species of the *Mangifera* genus have been shown to contain compounds with known anti-inflammatory properties such as quercetin, mangiferin, gallic and ellagic acids,

and rhamnetin, shown in Figure 3 (Masibo and He 2008). Numerous animal studies have confirmed that extracts of *M. indica* possess immunomodulatory properties, but these trials use extracts containing a large number of chemical entities and fail to link a specific compound or compounds to the observed beneficial effects (Rajendran, et al. 2013, Oluwole & Esume 2014, Márquez, et al. 2010). Any given plant species can contain hundreds of chemical entities, dozens of which may be unique to that particular plant genus (Atakan 2012).

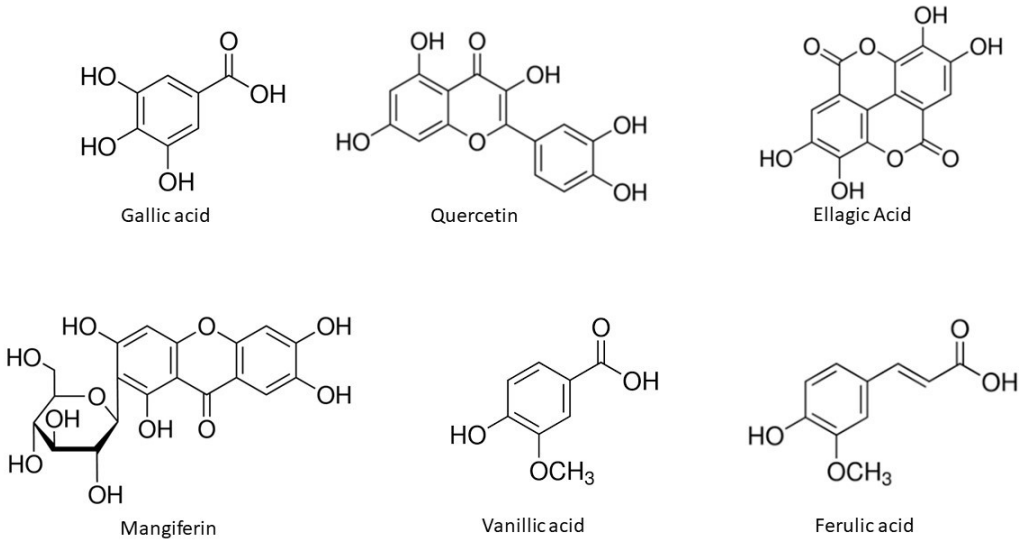

Figure 3. Known Immunomodulatory Compounds Found in *Mangifera* Genus

Gallic acid, quercetin, ellagic acid, mangiferin, vanillic acid, and ferulic acid are among the many known immunomodulatory compounds found in *Mangifera* species.

Bioassay-guided Fractionation Technique

Numerous analytical chemistry techniques exist for isolating and identifying compounds in a mixture of molecules. The identification of a cause-and-effect relationship between a specific compound and the observed immunomodulatory properties of a mixture of compounds is preferred over identifying all of the compounds in that mixture. A bioassay-guided fractionation of the mixture is one way to potentially identify a specific compound and definitively associate that compound with the bioactivity of the mixture (Weller 2012). Starting with the complex mixture, an initial fractionation is conducted, breaking the mixture of compounds into a set of mixtures, each containing fewer compounds than the original complex mixture. Each one of these fractions is tested for the bioactivity of interest. Fractions that exhibit bioactivity are then further fractionated, and the process is repeated until a single compound remains. That compound is then identified using analytical chemistry techniques. One important limitation of this technique is that, often, there is no single compound in the mixture that exhibits the bioactivity of that mixture. Instead, the observed bioactivity of the mixture is due to a combination of active compounds in that mixture.

Based on the potential benefit of identifying a cause-and-effect relationship between a single molecule and the immunomodulatory properties that have been previously observed in *Mangifera* species extracts, this project utilized bioassay-guided fractionation with the hypothesis that a single molecule is responsible for the immunosuppressive effects observed and able to be stably isolated and characterized.

MATERIALS AND METHODS

Plant Extract

The Guangxi Medicinal Botanical Gardens in Nanning, China provided the plant extract in collaboration with the Tennessee Center for Botanical Medicine Research (TCBMR) at MTSU. The extract used in this project is designated as 23B, and it is the ethyl acetate extract of the leaves and branches of *Mangifera persiciformis*.

Reagents

Dexamethasone (Dex), dimethyl sulfoxide (DMSO), phorbol 12-myristate 13-acetate (PMA), and RPMI 1640 culture medium were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS), 100X penicillin/streptomycin solution, and 32-63-micron ASTM reagent grade silica gel were purchased from Fisher Scientific (Pittsburgh, PA, USA). Alamar Blue was purchased from Life Technologies (Grand Island, NY, USA). ELISA kits and associated reagents were obtained from R&D Systems (Minneapolis, MN, USA). Bovine serum albumin (BSA) was obtained from EMD Millipore (Billerica, MA, USA).

Fractionation Using Flash Chromatography

Initial fractionation of the plant extract was conducted on a Teledyne CombiFlash Rf+ system. To prepare the extract for fractionation, 497.5 mg of the extract was weighed and dissolved in minimal dichloromethane. In a separate container, 5.002 g of flash grade silica gel was suspended in minimal dichloromethane to make a slurry. Once the silica gel was suspended in a slurry, the dichloromethane extract solution was slowly added and stirred. After thorough mixing, this solution was dried under light airflow. When the silica/sample mixture was completely dried, it was added to a 5 g silica gel sample cartridge. This cartridge was used in conjunction with a 40g preloaded silica column.

A stepwise gradient was conducted using hexane, ethyl acetate, and methanol for the mobile phase solvents (Table 1). The gradient began with 100% hexane. Then, by increments of 20%, ethyl acetate was added until an elution of 100% ethyl acetate was used. At each step, 200 mL of eluent was collected in a labelled collection container. Each of these collections constitutes a "fraction." Therefore, the first fraction was collected in 100% hexanes. The second was collected in 20/80 ethyl acetate/hexanes. The third fraction was collected in 40/60 ethyl acetate/hexanes, and so on. At the sixth fraction, the mobile phase was 100% ethyl acetate. The process was then repeated by adding methanol in increments increasing by 20% until the final step of 100% methanol. This process resulted in eleven 200 mL fractions of the extract.

Table 1. Elution Solvent and Recovery Amounts.

The elution solvent and recovery amounts for the fractions yielded from initial flash chromatography of 23B extract are shown. Hexane is abbreviated as "hex," ethyl acetate is abbreviated as "EA," and methanol is abbreviated at "Met."

Fraction	Solvent	Amount Recovered (mg)
1	100 hex	3.6
2	20/80 EA/hex	28.1
3	40/60 EA/hex	34.6
4	60/40 EA/hex	27.1
5	80/20 EA/hex	27.4
6	100 EA	16.4
7	20/80 Met/EA	171.6
8	40/60 Met/EA	34.4
9	60/40 Met/EA	12.2
10	80/20 Met/EA	8.3
11	100 Met	6.4

These 11 fractions were screened for immunomodulatory activity in THP-1 cells, and Fraction 4 was found to be of interest based on its immunosuppressive properties observed via a cytokine ELISA and the viability of the cells after treatment with the extract, as determined with an Alamar Blue Cell Viability assay, as described below.

Thin layer chromatography (TLC) of Fraction 4 was performed. The TLC was done on glass plates coated with silica gel (Fisher Scientific, Pittsburgh, PA). The TLC was conducted in a 1:1 ethyl acetate/hexanes solution. The TLC plates were analyzed with 254 nm UV light and then stained with phosphomolybdic acid. Three distinct spots were observed from Fraction 4 (Table 2). Further fractionation of Fraction 4 was done via manual flash chromatography. Manual flash chromatography was conducted using 32-63 µm ASTM reagent grade silica gel as the stationary phase. The mobile phase started at 1:10 ethyl acetate/hexanes. The proportion of ethyl acetate was slowly increased until a 100% ethyl acetate fraction was collected. This was followed by a methanol rinse of the column. Elution of compounds at each solvent concentration was monitored via (TLC) in a 1:1 ethyl acetate/hexanes solution. The TLC plates were analyzed with 254 nm UV light and then stained with phosphomolybdic acid. When an isolated spot appeared on the TLC plate, fractions containing this spot were recovered and consolidated.

Further Fractionation of the Extract with HPLC

Further fractionation of F4-2 was done via reverse-phase high performance liquid chromatography (RP HPLC) using a Waters 1525 binary HPLC pump in conjunction with a waters 2489 UV/visible detector. A constant gradient with a flow rate of 4 mL/min was used. A Thermo Scientific 250 x 10mm Hypersil Gold aQ C18 column was utilized, with a 5µm particle size and a pore size of 175Å. The separation protocol began with a mobile phase of 70/30 water/acetonitrile and progressed linearly to 30/70 water/acetonitrile over 30 min. This was followed by a brief organic rinse (95% acetonitrile) and an aqueous rinse

(95% water). Absorbance was measured at 254 nm. This wavelength was chosen after a full-spectrum analysis of the sample using a SpectraMax M2e microplate reader revealed the only major absorbance wavelengths in the sample to be in the UV range.

Maintenance and Preparation of THP-1 Cell Line

The continuous human monocyte cell line, THP-1, was used for the Alamar Blue cell viability assays and the TNF-α cytokine ELISAs. Once differentiated into macrophage-like cells, these cells express surface CD14 and TLR4, making them sensitive to stimulation with bacterial lipopolysaccharides (LPS) (Aldo, et al. 2013). The cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (complete medium), and they were incubated at 37°C with 5% CO_2. Prior to each assay, the cells were counted with a hemocytometer and adjusted to a concentration of $5x10^5$ cells/mL by centrifugation at 500 x *g* for 5 min and resuspending in complete medium at the desired concentration. The THP-1 monocytes were differentiated into adherent, macrophage-like cells prior to each assay. This was done chemically by adding phorbol 12-myristate 13-acetate (PMA) to the complete medium upon resuspension of the counted cells to a concentration of 100 nM. Then, 500 µL of cells were added to each well of a 24-well plate, and the cells were allowed to differentiate for approximately 48h before each assay. Immediately prior to each assay, the cells were washed with incomplete medium to remove excess PMA and dead cells. After rinsing and aspirating the medium, 500 µL of complete RPMI medium was added to each well.

LPS Stimulation Assay

The THP-1 cells were tested with a range of LPS concentrations to assess which amount was optimal for the bioassays. It was determined that 20 ng/mL of LPS was the best concentration for yielding TNF-α levels that can be reproducibly detected in the ELISA, according to the ELISA manufacturer's protocol. Dexamethasone, a synthetic glucocorticoid, suppresses cytokine production at this concentration of LPS in THP-1 cells (Steer, et al. 2000). For this reason, it was used as a positive control for immunomodulation throughout this study.

To prepare the extract fractions for bioassays, the samples were weighed and suspended in dimethyl sulfoxide (DMSO) to a concentration of 10 mg/mL. To test for cell viability and cytokine production in the presence of the plant extract treatments, the THP-1 cells were prepared and differentiated as previously described in a 24-well plate. After rinsing with incomplete RPMI, the cells were pretreated with fractions of the plant extract at varying concentrations (25 µg and 50 µg) or 1µM dexamethasone for 1h, then they were stimulated with 20 ng/mL of LPS for 4h (Park, et al. 2016). Each treatment was administered in duplicate wells.

Cell Viability and Cytokine Response Measurements

After the 4h LPS stimulation, the supernatant of each well was collected and stored in a labeled 1.5 mL microfuge tube at -80°C for later use in the cytokine ELISA. After removing the supernatant from the 24-well plate, 450 µL of complete medium were added

to each well. Then, 50 μL of 10X Alamar Blue cell viability solution were added to each well, and the plate was incubated at 37°C overnight. Viability was measured by relative fluorescence units (RFU) at Ex 560 nm and Em 590 nm. Viability was reported as a percentage compared to an untreated control. The collected supernatant was measured for cytokine response using R&D Systems human TNF-α ELISA kits and reagents per the manufacturer's protocol. The final isolated compound was tested in this manner three times, in technical duplicates each time. The Student's t-test was used to assess significance of the amount of TNF-α produced in LPS-treated cells for compared with F4-2b or dexamethasone-treated cells for each of the three independent ELISA trials. Statistical significance was determined at $p < 0.05$.

Magnetic Resonance Spectroscopy (NMR)

NMR spectra were obtained using a 500 MHz FT-NMR model ECA-500 JEOL (Peabody, MA) purchased with funding provided by the National Science Foundation. ^{13}C-NMR and ^{1}H-NMR were conducted, and chemical shifts are reported in parts per million (ppm) in reference to tetramethylsilane (TMS).

RESULTS

The ethyl acetate extract from the leaves and branches of *M. persiciformis* was first fractionated via automated flash chromatography, producing eleven fractions. Fractions 2-11 from the initial CombiFlash fractionation of 23B were tested for TNF-α inhibition and cytotoxicity. Fractions 4, 5, and 6 suppressed the production of TNF-α, as determined via a cytokine ELISA (Figure 4). However, Fractions 5 and 6 were found to be toxic, and only Fraction 4 possessed both qualities of inhibiting the production of TNF-α and maintaining a cell viability of greater than 90% (Figure 4 and Figure 5).

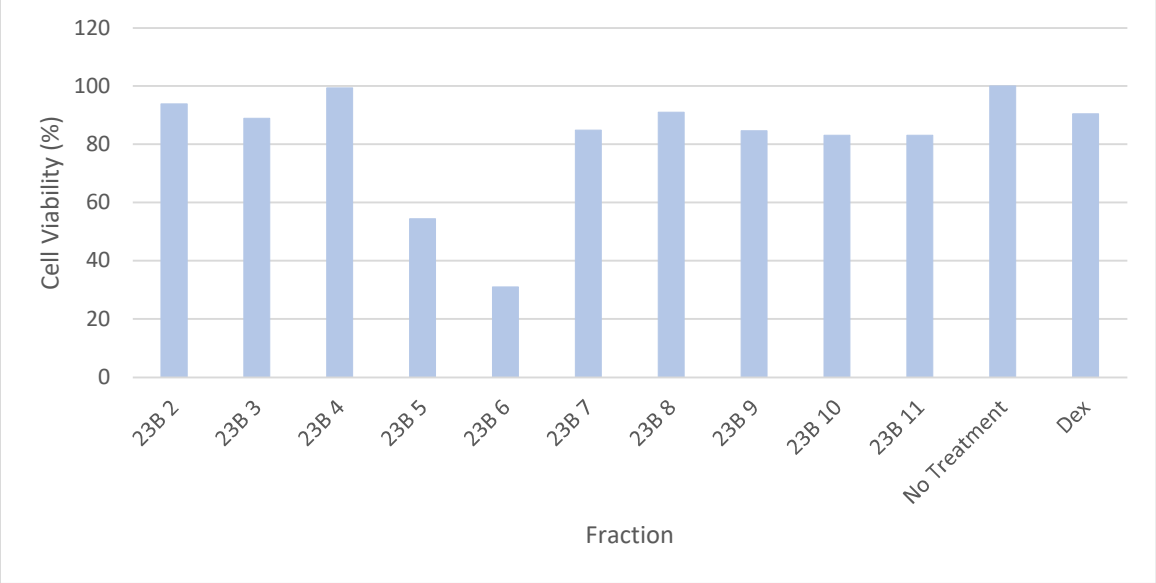

Figure 4. Percent cell viability of THP-1 cells treated with 23B fractions.

Fraction 4 maintained a cell viability of >90%, as measured using the Alamar Blue cell viability assay. However, fractions 5 and 6 were both toxic and yielded viabilities <60%.

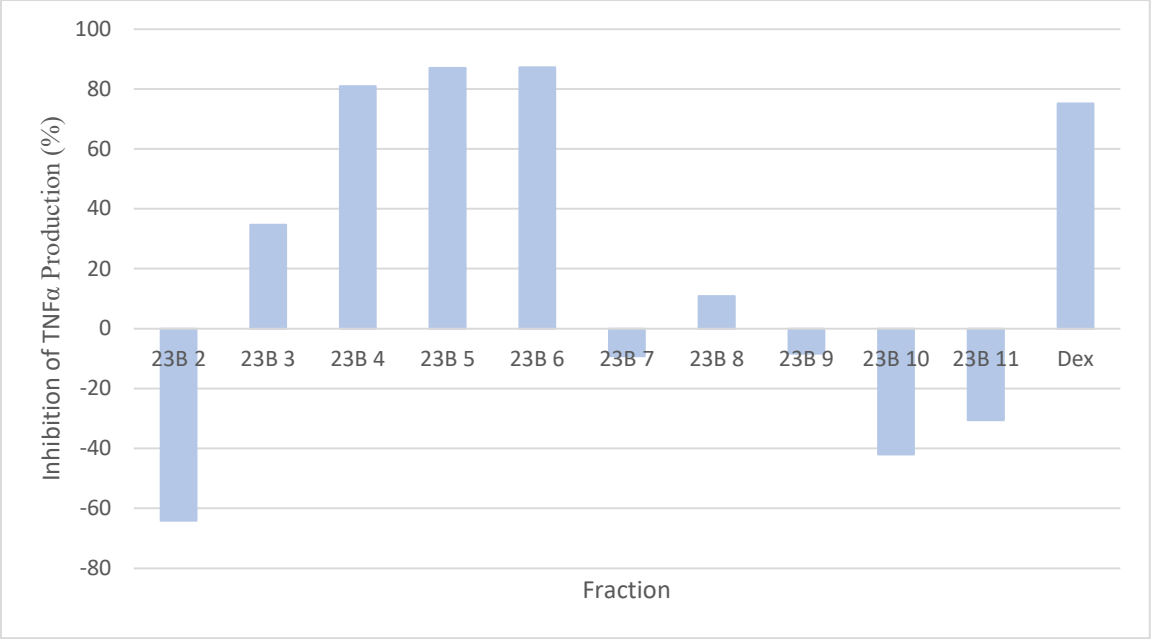

Figure 5. Percent inhibition of TNF-α by fractions of 23B.

Fractions 4, 5, and 6 all suppress the production of TNF-α. Levels of TNF-α production were measured using cytokine ELISAs after LPS stimulation assay.

Due to its immunosuppressive activity and low toxicity in THP-1 cells, Fraction 4 was further fractionated by manual flash chromatography. Fractions were screened with TLC, and fractions were recovered when a distinct spot was present on the TLC plate. Two spots were recovered and isolated using this method (Table 2). These recovered fractions were labelled as F4-1 and F4-2.

Table 2. Rf Values and Amount Recovered from F4.

TLC of Fraction 4 showed 3 distinct spots. The first and least polar of these spots was not recovered during manual flash chromatography. The other two spots were observed and isolated.

Rf value in 1:1 EA/Hex	**Amount recovered via flash chromatography**
Spot 1: 0.57	not recovered
Spot 2: 0.36	F4-1 : 2.5mg
Spot 3: 0.19	F4-2: 18.6mg

Due to the amount of material available, F4-1 was not investigated. TLC analysis of fraction F4-2 in a solution of 4:1 ethyl acetate/hexanes revealed that the spot observed was not a purely isolated compound but instead consisted of at least two compounds with Rf values of 0.52 and 0.62.

During HPLC of Fraction F4-2, fractions were collected during peaks of absorbance at 254 nm. Two such fractions were collected. These fractions were labeled "F4-2a" and "F4-2b" with retention times (RT) of 6.0 min and 8.0 min, respectively (Figure 6). Fraction F4-2b was analyzed using TLC in a 4:1 ethyl acetate/hexanes solvent solution. It was found to consist primarily of a single compound with an Rf value of 0.64. From the HPLC, 3.65 mg of F4-2b was recovered. A 1.25 mg sample of this was reserved for use in the bioassays, and the remaining 2.4 mg was used for NMR analysis.

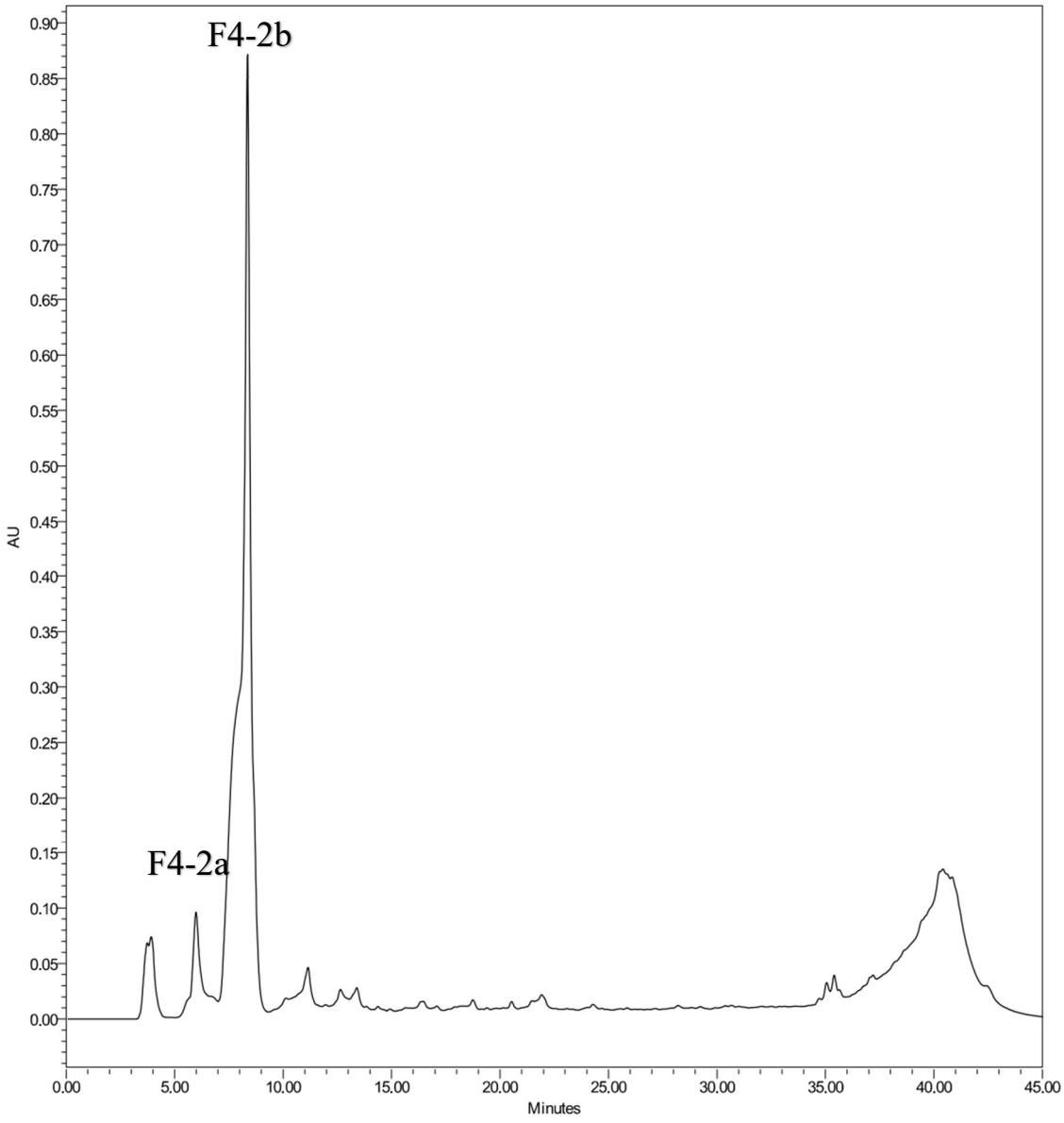

Figure 6. HPLC chromatograph of F4-2

Two compounds were recovered during HPLC of fraction F4-2. Compound F4-2b was found to be immunomodulatory and was further analyzed via NMR spectroscopy.

The compound F4-2b was screened for TNF-α inhibition and cell viability. Duplicate wells of cells were treated for 1h with either medium alone, F4-2b, or

dexamethasone as the positive control for inhibition of TNF-α, followed by treatment with LPS for 4h. The supernatants from these wells were tested in technical duplicates in the cytokine ELISAs. This process was conducted three times. The compound F4-2b was found to suppress the production of TNF-α by 71.38%, 80.13%, and 84.55% in three independent trials in LPS-stimulated THP-1 cells compared to an LPS only-treated control (Appendix). Levels of TNF-α were significantly decreased ($p < 0.005$, 0.01, and 0.01) in F4-2b-treated cells compared to LPS only-treated cells in three respective trials (Appendix) and cell viability of the F4-2b-treated cells was maintained at greater than 90%. The positive control treatment with dexamethasone (1 μM) also resulted in significant inhibition of TNF-α with only one of the three trials being significantly lower than treatment with F4-2b, demonstrating that F4-2b was as effective as the steroid in reducing cytokine levels, while maintaining a high level of cell viability.

After the bioassays, the remainder of F4-2b was analyzed using NMR spectroscopy. ^{1}H-NMR (Figure 7) and ^{13}C-NMR (Figure 8) revealed shifts that indicate aromatic carbons, a carbonyl, and a methyl ether are present in compound F4-2b.

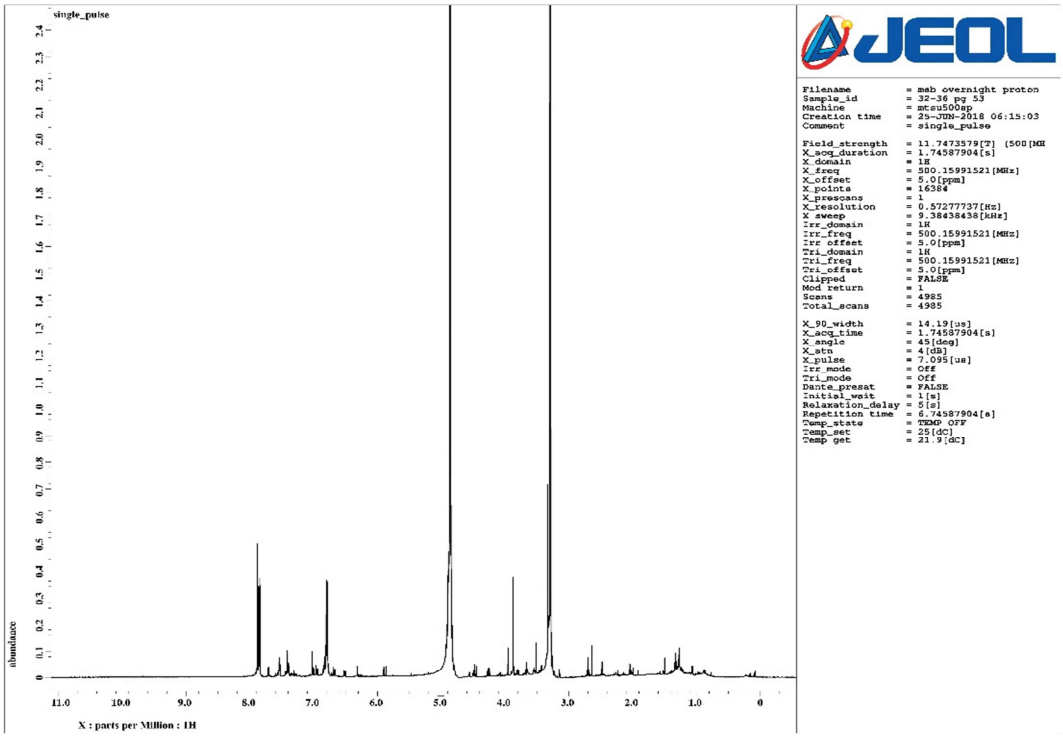

Figure 7. ^{1}H-NMR of F4-2b.

Rf 0.64 [4:1 ethyl acetate/hexanes]; ^{1}H-NMR(500 MHz, methanol-d): a methoxy is indicated at 3.8 ppm, and aromatic hydrogens at 6.8 ppm and 8.0 ppm.

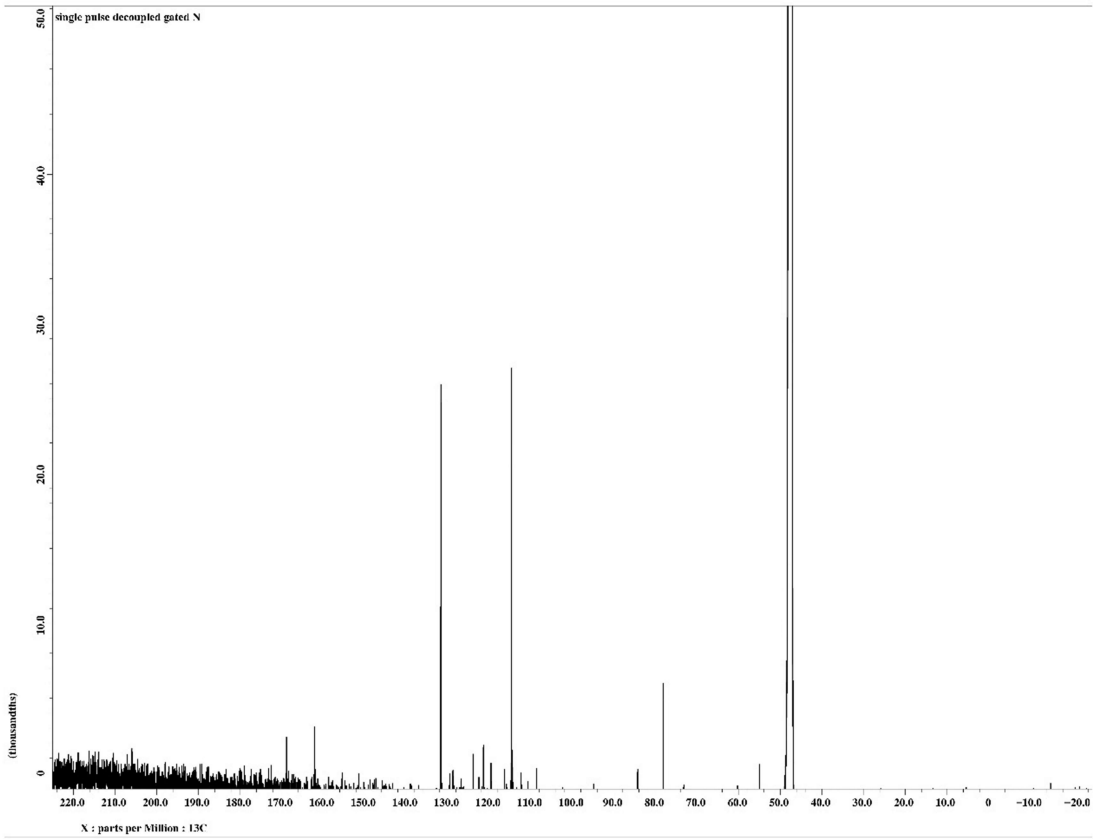

Figure 8. ^{13}C-NMR of F4-2b

Rf 0.64 [4:1 ethyl acetate/hexanes]; ^{13}C-NMR(500 MHz, methanol-d): a methoxy carbon is at 78 ppm, the signals at 115 ppm, 132 ppm, and 162 ppm likely represent aromatic carbons, and the signal at 170 ppm is a carbonyl.

DISCUSSION

Inflammation is a normal part of a functional immune response. It is an essential element of the body's ability to combat infection and repair damage. However, inappropriately stimulated inflammation is the basis of many diseases, such as Crohn's Disease, atopic dermatitis, and inflammatory bowel disease. Additionally, prolonged or chronic inflammation greatly increases the likelihood of cancer development (Coussens & Werb 2002). The current anti-inflammatory therapies have many shortcomings. Corticosteroids and NSAIDs cause unwanted side effects. TNF blockers are very expensive and potentially have serious side effects, as well. There exists in traditional Chinese medicine a vast library of potentially useful immunomodulatory compounds. Compounds isolated from natural products have already contributed greatly to the field of medicine, and these therapies will continue to do so as more research is done to isolate and characterize them.

This project utilized a bioassay-guided fractionation pathway previously established by the TCBMR at MTSU. The combination of cytokine ELISA and cell viability assay using the same cells is a useful technique for quickly identifying which fractions are immunomodulatory and which ones may be suppressing the production of TNF-α by damaging or killing the cells. In the case of this project, the fractions that stimulated the most potent inhibition of TNF-α production were also the most toxic.

Bioassay-guided fractionation is most useful when a single molecule is responsible for enough of the studied phenomenon that the effectiveness of the raw extract can be attributed to that molecule (Tu, et al. 2019). One risk of bioassay-guided fractionation is

the likelihood of a desired characteristic (i.e., immunomodulatory, anti-fungal, or anti-viral properties) of a plant extract being attributed to multiple molecules working in tandem. In such cases, activity of the fractions can be diminished or disappear once the molecules of interest are separated from each other into different fractions. Another obstacle with bioassay-guided fractionation is the amount of original material required to yield enough isolated product for chemical and biological analysis. Depending on the origin of the plant material, it can be difficult to obtain enough material for meaningful biological and chemical analysis of a final, isolated product.

The identification of immunomodulatory compounds in *Mangifera* species is well-documented. Mangiferin, gallic acid, vanillic acid, ellagic acid, quercetin, and benzoic acid are just a few of the many immunomodulatory molecules that have been found in extracts of *Mangifera* (Maldonado-Celis, et al. 2019). The compound isolated in this project was not isolated in a pure quantity sufficient for identification. The small number of carbon signals suggest that it could be a small phenolic compound similar to vanillic or gallic acid. The results of this study were promising. The NMR spectra of F4-2b do not match the spectra of any of the major immunomodulatory compounds known to exist in *Mangifera* species such as mangiferin, quercetin, or ellagic acid. However, future studies with more purified material will be required in order to identify the structure of F4-2b. Additionally, further future studies could elucidate the mechanism of action of F4-2b and determine which inflammatory pathways are suppressed by its activity.

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APPENDIX

Appendix: Table summarizing data and statistical analyses for treatment of LPS-stimulated THP-1 cells with fraction F4-2b and controls.

ELISA 1			**ELISA 2**			**ELISA 3**	
Duplicate Samples	Replicates TNF (pg/mL)		Duplicate Samples	Replicates TNF (pg/mL)		Duplicate Samples	Replicates TNF (pg/mL)
F4-2b	758.56		F4-2b	57.3		F4-2b	266.72
	736.07			58.31			248.69
F4-2b	590.98		F4-2b	81.55		F4-2b	124.56
	686.88			75.07			111.04
Avg	693.1225		Avg	68.0575		Avg	187.7525
Stdev	74.3832716		Stdev	12.1375488		Stdev	81.296463
SEM	37.1916358		SEM	6.06877442		SEM	40.6482315
LPS	2678.99		LPS	413.53		LPS	1507.77
	2048.9			380.49			1437.22
LPS	2414.28		LPS	287.43		LPS	1085.38
	2544.36			288.92			831.25
Avg	2421.6325		Avg	342.5925		Avg	1215.405
Stdev	270.97263		Stdev	64.2702318		Stdev	315.785134
SEM	135.486315		SEM	32.1351159		SEM	157.892567
Dex	208.17		Dex	69.49		Dex	68.63
	241.59			70.02			60.24
Dex	206.09		Dex	63.8		Dex	67.26
	212.74			63.48			54.54
Avg	220.14		Avg	65.7666667		Avg	60.68
Stdev	18.8714732		Stdev	3.68696804		Stdev	6.37140487
SEM	8.265015		SEM	1.76976635		SEM	3.27352352
F42b:LPS % Inhibit	71.3778825		F4-2b:LPS % Inhibit	80.1345622		F4-2b:LPS % Inhibit	84.5522686
Dex:LPS % Inhibit	90.9094382		Dex:LPS % Inhibit	80.8032381		Dex:LPS % Inhibit	95.0074255
STDEV	F4-2b		LPS		Dex		
	Avg	Std Dev	Avg	Std Dev	Avg	Std Dev	
ELISA 1	693.1225	74.3832716	2421.6325	270.97263	220.14	18.8714732	
ELISA 2	68.0575	12.1375488	342.5925	64.2702318	65.7666667	3.68696804	
ELISA 3	187.7525	81.296463	1215.405	315.785134	60.68	6.37140487	
T test *p*	F4-2b:LPS	Dex:LPS	F4-2b:Dex				
ELISA 1	0.00114496	0.00059105	0.00163076				
ELISA 2	0.00547392	0.00283604	1.74496345				
ELISA 3	0.00327914	0.00512039	0.05034693				