HHS Public Access

Author manuscript

J Neuroimmunol. Author manuscript; available in PMC 2017 January 15.

Published in final edited form as:

J Neuroimmunol. 2017 January 15; 302: 10–19. doi:10.1016/j.jneuroim.2016.11.012.

Natural product HTP screening for evidence of attenuated cytokine-induced neutrophil chemo attractants (CINCs) and NO2− in LPS/IFNγ activated glioma cells

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Abstract

Chronic or acute central nervous system (CNS) inflammation is carried out by glial cells, which can contribute to neurological injuries associated with head trauma, stroke, and infection, Parkinson's or Alzheimer 's disease. The process of aging combined with inflammation are also risk factors for developing glioblastoma multiforme (GBM) as well as perpetuating its malignant aggression. With growing public curiosity in complementary and alternative medicines (CAMs), in this work we conduct a high throughput (HTP) screening of >1400 natural herbs, plants and over the counter (OTC) products for anti-inflammatory effects on lipopolysaccharide (LPS)/interferon gamma (IFNγ) activated C6 glioma cells. Validation studies suggest a pro-inflammatory profile mediated by LPS [3μg/ml/IFNγ 3ng/ml] is consistent with elevation [> 8.5 fold] of MCP-1 and cytokine-induced neutrophil chemo-attractants (CINC) 1, CINC 2a and CINC3. The data show no evidence of changes to the following, IL-13, TNF-a, fracktaline, leptin, LIX, GM-CSF, ICAM1, L-Selectin, activin A, agrin, IL-1α, MIP-3a, B72/CD86, NGF, IL-1b, MMP-8, IL-1 R6, PDGF-AA, IL-2, IL-4, prolactin R, RAGE, IL-6, Thymus Chemokine-1, CNTF,IL-10 or TIMP-1. The data also show a LPS/IFNγ mediated rise in iNOS and NO2− as often reported. A HTP screening was conducted, where we employ an *in vitro* efficacy index (*i*EI) defined as the ratio of toxicity (LC_{50})/anti-inflammatory potency (IC_{50}) to ensure biological effects were occurring in fully viable cells (ratio > 3.8). Using NO2− as a guideline molecule, the data show that 1.77 % (25 of 1410 tested) (at <250μg/ml) had anti-inflammatory effects with an *i*EI ratio >3.8. These include reference drugs (hydrocortisone, dexamethasone N6-(1-iminoethyl)-L-lysine and NSAIDS : diclofenac, tolfenamic acid), a histone deacetylase inhibitor (apicidin) and the following natural products; Ashwaganda (*Withania somnifera*), Elecampagne Root (*Inula helenium*), Feverfew (*Tanacetum parthenium*), Green Tea (*Camellia sinensis*), Turmeric Root (*Curcuma Longa*) Ganthoda (*Valeriana wallichii*), Tansy (*Tanacetum vulgare*), Maddar Root (*Rubia tinctoria*), Red Sandle wood (*Pterocarpus santalinus*), Bay Leaf (*Laurus nobilis*, Lauraceae), quercetin,

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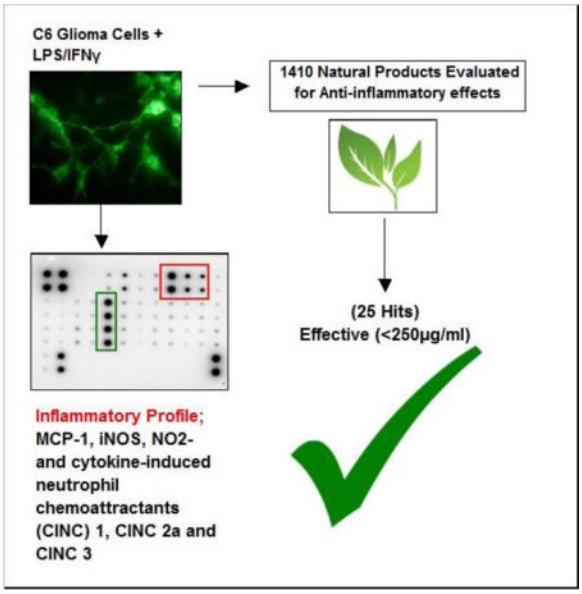
Conflict of Interest

N/A

cardamonin, fisetin, EGCG, biochanin A, galangin, apigenin and curcumin. The herb with the largest ƒEI was Ashwaganda where the IC_{50}/LC_{50} was 11.1/>1750.0 μg/mL, and the compound with the greatest ƒEI was quercetin where the IC_{50}/LC_{50} was 10.0/>363.6 ug/mL. These substances also downregulate the production of iNOS expression and attenuate CINC-3 release. In summary, this HTP screening provides guideline information as to efficacy of natural products that in the long term, could prevent inflammatory processes associated with neurodegenerative disease and aggressive glioma tumor growth.

Graphical abstract



Introduction

CNS Inflammation, Glial cells and Neurodegeneration

Age related central nervous system (CNS) degenerative diseases such as Parkinson's (PD) and Alzheimer's disease (AD) are becoming significant global health concerns. Inflammatory processes occurring within CNS (by glial cells) contribute vulnerability by initiating vascular damage (Doyle et al., 2015, Weaver et al., 2010) neurological insult (Bodnar et al., 2015, Garcia et al., 2016) and destruction to the blood brain barrier (BBB). (Adelson et al., 1998) While CNS glial cells exert an influential role in neurodevelopment, neuronal homeostasis and CNS detoxification, on the flip side, detrimental inflammatory processes can arise from head trauma (Lopez-Rodriguez et al., 2015), ischemia (Li et al., 2015c) infection (Ben Haim et al., 2015a) lysosomal storage diseases (Rama Rao and Kielian, 2015) and protein aggregates of amyloid β (Aβ) (Ben Haim, Carrillo-de Sauvage, 2015a) and α-synuclein A53T (Ben Haim, Carrillo-de Sauvage, 2015a, Yang et al., 2015) common to AD and PD, respectively. Once neurodegeneration ensues, reactive gliosis (Mohn and Koob, 2015) circumscribing degenerative neurons can occur due to debris generated as danger-associated molecular patterns (DAMPs), (Frank et al., 2016, Kigerl et al., 2014) leading to a cyclic perpetuated release of pro-inflammatory neurotoxic cytokines

(Hammond et al., 2015, Mohn and Koob, 2015). Chronic CNS inflammation can manifest itself systemically, where high elevated cytokines are often reported in the cerebral spinal, synovial or serum in patients with PD (Bessler et al., 1999) or AD (Blum-Degen et al., 1995, Brodacki et al., 2008).

CNS Inflammation, Glial Cells and Malignancy

While aging and chronic inflammation are associated with major neurodegenerative disorders, both events are also risk factors for malignant glioblastoma multiforme (GBM), and its radiotherapeutic resistance.(Li and Liu, 2015) Glial tumors originate from sites of chronic irritation/inflammation and once formed, perpetuate release of pro-tumor cytokines as means to drive tumor cell proliferation, resistance, and immune escape (Salazar-Ramiro et al., 2016). Glioma cells have enormous capacity to release chemokines such as MCP-1, which traffic recruitment of tumor associated monocytes (TAMS) (Leung et al., 1997) to infiltrate the glioma tumor bed (Lindemann et al., 2015, Polyzoidis et al., 2015). These processes then drive aggressive malignancies common to both glioblastomas and astrocytomas (Liang et al., 2008, Lin et al., 2013).

There is growing public awareness about the potential use of complementary and alternative medicine (CAMS) to reduce risk of chronic disease. In this study, we use a high through put (HTP) screening procedure to test 1410 natural products (herbs, seeds, roots, leaves, stems) which are available to consumers worldwide as OTC nutraceuticals. The study design in employs the use of a C6 glioma cell line to conduct the HTP screenings. C6 cells are of malignant origin and widely used to investigate neuro inflammation relevant to brain disorders, depression, AD, PD, schizophrenia (de Souza et al., 2013, Kawashima et al., 2008, Lykhmus et al., 2015) defects in iron metabolism (di Patti et al., 2004) glutamate uptake (dos Santos et al., 2006) multiple sclerosis (MS)(Harzheim et al., 2003) as well as therapies and processes associate with aggressive malignant tumor growth and invasion (Adach-Kilon et al., 2011, Li et al., 2016, Liau et al., 1998, Pineda et al., 2005). Glioma tumor growth is advanced by an inflammatory microenvironment involving inducible nitric oxide synthase (iNOS) leading to enhanced vasodilation, proliferation (Munoz-Fernandez and Fresno, 1993) migration (Yeh et al., 2012) and the rapid growth of GBMs (Cobbs et al., 2003).

In this study, we explore a large number of nutraceutical products for evidence of capacity to inhibit inflammation within activated glioma. Using stringent controls to account for therapeutic effects in fully viable cells, the data show that only 25/1410 tested have confirmed anti-inflammatory effects with a relatively high level of confidence.

Methods and Materials

Hanks Balanced Salt Solution, (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES), ethanol, sufanilamide, 96 well plates, general reagents and supplies, were all purchased from Sigma-Aldrich (St Louis, MO) or VWR (Radnor, PA). Imaging probes were purchased from Life Technologies (Grand Island, NY). Natural products were purchased from Frontier Natural Products Co-op (Norway, IA), Monterey Bay Spice Company (Watsonville, CA), Mountain Rose Herbs (Eugene, OR), Mayway Traditional Chinese Herbs

(Oakland, California), Kalyx Natural Marketplace (Camden, NY), Futureceuticals (Momence, IL), organic fruit and vegetable market (New Leaf, Tallahassee, FL), Florida Food Products Inc. (Eustis, FL), Patel Brothers Indian Grocery (Tampa, FL) and OpilGold from AgingSciences LLC (Wayland, MA). Elisa kits and cytokine antibody arrays were purchased from Assay Biotech (Sunnyvale, CA) and Raybiotech (Norcross, GA).

Cell Culture

C6 cells were purchased from American Type Culture Collection (Manassas, VA). Cells were brought up according to the manufacturers instruction, then sub cultured in DMEM high glucose media [glucose 4500mg/L] containing 5% FBS, 4 mM L-glutamine, and penicillin/streptomycin (100 U/0.1 mg/ml). Culture conditions : maintained at 37°C in 5% CO_2/atmosphere. For experiments, plating media consisted of DMEM (minus phenol red) [glucose 4500mg/L], 2.5% FBS and penicillin/streptomycin (100 U/0.1 mg/ml). LPS O111:B4 was prepared in HBSS at 1 mg/ml and stored at −20°C. Rat Interferon gamma (IFNγ) was prepared according the manufacturer's instructions and aliquoted in siliconized micro centrifuge tubes, then stored at −20°C. For experiments, LPS/IFNγ was added to the culture media at a working concentration of 3μg/3ng per ml.

Herbal, Compound and Drug Preparations

All natural chemicals and reference drugs were dissolved in DMSO [5–20mg/mL] and crude herbs in absolute ethanol [50mg/ml] after being diced, macerated and powdered then stored at −20°C. All dilutions were prepared in sterile HBSS + 5 mM HEPES, adjusted to a pH of 7.4 and solvent concentration of DMSO or Etoh was less than 0.5%.

Cell Viability

Cell Viability was quantified using resazurin [7-Hydroxy-3H-phenoxazin-3-one 10-oxide] (Alamar Blue) indicator dye. (Evans et al., 2001) A working solution of resazurin was prepared in sterile HBSS – phenol red (0.5 mg/ml) and added (15% v/v) to each sample. Samples were returned to the incubator for 2–4 hr, and reduction of the dye by viable cells (to resorufin, a fluorescent compound) was quantitatively analyzed using a Synergy HTX multi-mode reader (Bio-Tek, Winooski, VT) with settings at [550/580], [excitation/emission].

Nitrite (NO2−,)/iNOS Expression

Quantification of nitrite (NO2−) was determined by using the Greiss reagent. (Cendan et al., 1996) The Greiss reagent was prepared by mixing an equal volume of 1.0% sulfanilamide in 10% phosphoric acid and 0.1% N-(1-naphthyl)-ethylenediamine in deionized water, then added directly to the cell supernatant (experimental media consisting of DMEM – phenol red) and incubated at room temperature for 10 min. Controls and blanks were run simultaneously, and subtracted from the final value to eliminate interference. Samples were analyzed at 540 nm on a Synergy HTX multi-mode reader (Bio-Tek, Winooski, VT)

For iNOS protein expression, cells were fixed in 4% paraformaldehyde/permeabilized in 0.2% triton X-100 in phosphate buffered saline (PBS) and incubated with anti-iNOS, N-Terminal antibody produced in rabbit for 24 hours at 4°C in a casein blocking buffer.

Samples were washed in PBS, then incubated with anti-rabbit Alexa Fluor® 488 conjugate for two hours at RT. Samples were photographically imaged using a fluorescent/inverted microscope, CCD camera and data acquisition using ToupTek View (TouTek Photonics Co, Zhejiang, P.R. China).

High-Through Put Methods—*In vitro* HTP screenings of natural plants can be informative, but require a number of critical controls to eliminate interfering variables such as inherent variation in pH, solvents and most important, cytotoxicity. In the case of natural products, cell death becomes a pivotal issue given the dual property of many natural products to incur both anti-cancer and anti-inflammatory effects through similar pathways for example the phosphorylation of extracellular signal-regulated kinase (ERK), c-jun NH2-terminal kinase (JNK) phosphorylation and p38 MAPK NF-κB (Amirghofran, 2012, Huang et al., 2007, Pan et al., 2015, Zhang et al., 2015). This aspect becomes important, in particular when using astrocyte cell lines of malignant and immune origin, such as rat C6 glioma cells. For this reason, we construct and use an *in vitro* efficacy index (*I*EI) defined as LC_{50} (toxic concentrations)/IC_{50} (anti-inflammatory concentrations), to where a higher ratio establishes a greater confidence for the anti-inflammatory proponent with little interfering effects of cytotoxicity. The data from this study, reveal that a very small percentage of natural compounds, at low concentration (<250μg/ml) have anti-inflammatory effects against LPS/IFNγ activation in C6 glioma cells at therapeutic concentrations comparable to NSAIDS.

Rat Cytokine Antibody Array

Rat Cytokine Antibody Arrays – (34 Targets/ab133992) (Abcam, Cambridge, MA) were used to profile the effects of LPS/IFNγ on C6 glioma cells. Each experiment was carried out in accordance with manufacturer's instructions. Detection of spots using chemiluminescence was acquired using a VersaDoc Imager/Quantity One software (Bio-Rad).

Cytokine-Induced Neutrophil Chemoattractant 3-ELISA

Sample cell supernatants were evaluated for release of CINC-3 using a CINC-3 (CXCL2) Rat ELISA Kit (ab155463) (Abcam, Cambridge, MA). Quantification of CINC-3 release was performed according to the manufacturer's guidelines. Data was quantified at 450 *nm* using a Synergy HTX multi-mode reader (Bio-Tek, Winooski, VT).

Data analysis

Statistical analysis was performed using Graph Pad Prism (version 3.0; Graph Pad Software Inc. San Diego, CA, USA) with significance of difference between the groups assessed using a one-way ANOVA, followed by Tukey post hoc means comparison test, or Student's t test. IC_{50}s and LC_{50}s were determined by regression analysis using Origin Software (OriginLab, Northampton, MA).

Results

Validation studies were conducted to establish dominant inflammatory molecules in LPS 3μg/ml/IFNγ 3ng/ml activated C6 glioma cells (Figure 1A,B), where significant changes in

CINC (1,2a and 3) release were observed. Release of NO2− was also significantly elevated in LPS/IFNγ treated cells vs. resting cells, then reduced by hydrocortisone and L-NIL (20μg/ml) (Figure 2A) the latter, without variation in cell viability (Figure 2B). This pattern coincided with iNOS protein expression, (Figure 2C), and 24 hour release of CINC-3 (Figure 2D) to the exception of L-NIL. These findings demonstrate that L-NIL appears to be target specific (enzyme inactivation of iNOS), offering little influence on the process of inflammation as a whole, unlike hydrocortisone which reduces both NO-2, iNOS and CINC3 release.

A high-through put screening of natural compounds consisted of product library containing ethanol extracts of 1) Plants: Seeds, Fruits, Veg etc. and herbs (commonly used Chinese, Egyptian, Indian and Organic grown in the United States) 2) Natural derived chemicals/ polyphenolics 3) Metabolic Substrates: Amino Acids, Vitamins and Energy intermediates such as organic acids and 4) reference NSAID and steroidal anti-inflammatory drugs. A primary screen was conducted to which all compounds were evaluated for reduction of NO2− in LPS/IFNγ activated C6 cells. Natural plant extracts were tested at a maximum concentration of 230μg/ml and compounds at a maximum of 92μg/ml for the initial preliminary screen (Figure 3A). Substances having an IC_{50} for NO2− inhibition at equal to or below these levels, were re-evaluated over a minimum of 6 dose dependent concentrations where LC_{50} (cytotoxicity) and IC_{50} (NO2−) where simultaneously determined (Figure 3B) and an *in vitro* efficacy index (*i*EI) established – LC_{50}/IC_{50}. The higher the ratio, the greater confidence in anti-inflammatory effects, relative to influence from cytotoxicity. All *i*EI values are presented in Table 1, which are provided with a matching scatterplot (Figure 4). Figure 5 shows the full NO2−/viability dose response data for some of the most effective compounds, where an optimal single point n=3, reflects supernatant evaluated for CINC3 by ELISA. These findings highlight the most effective anti-inflammatory natural compounds elucidated using this study model.

Discussion

In the CNS, there are numerous triggers which evoke inflammation in immunocompetent cells such as glia, with subsequent events leading to neurodegeneration as well as driving forces toward aggressive malignant glioblastoma. Outward manifestation of reactive gliosis/ neuro inflammatory processes are associated with head trauma (Lopez-Rodriguez, Acaz-Fonseca, 2015), ischemia (Li, Xu, 2015c) infection (Ben Haim, Carrillo-de Sauvage, 2015a) and neuronal lesions infiltrated with protein aggregates such as amyloid β (Aβ), neurofibrillary tangles (Ben Haim, Carrillo-de Sauvage, 2015a) α-synucleinA53T (Ben Haim, Carrillo-de Sauvage, 2015a, Yang, Wang, 2015) which are common to AD and PD pathologies, respectively. Sustained neuro-inflammatory processes can be perpetuated by dying cells which release DAMPs such as high mobility group box-1 (HMGB1)/capable of activating innate immunity to remove necrotic debris, then worsening the neurodegenerative process (Frank, Adhikary, 2016, Kigerl, de Rivero Vaccari, 2014). Reactive gliosis contributes to release of toxic molecules such as NO2−, growth factors (lipocalin-2) and chemokines including the CINCs (Lee et al., 2015, Lopez-Rodriguez, Acaz-Fonseca, 2015). CINCS are reportedly elevated as a consequence to ischemic (Wang et al., 2006) and vascular injuries (Weaver, Zhang, 2010), being coexpressed with GFAP, vimentin, synemin,

nestin (Hol and Pekny, 2015) and alphaB-crystallin which is present in multiple sclerosis lesions (Liu et al., 2015). In the case of glioblastoma multiforme (GBM), excessive inflammation can drive not only a genetic instabilities, but also tumor proliferation, resistance and immunological evasion (Lin, Zhang, 2013, Polyzoidis, Koletsa, 2015, Salazar-Ramiro, Ramirez-Ortega, 2016). Much of this occurs due to glioma release of chemokines such as MCP1 can traffic monocyte infiltration and docking to glioma, then capable of transforming to an M2 pro-angiogenic phenotype which propels malignant aggressively (Leung, Wong, 1997, Liang, Bollen, 2008, Lindemann, Marschall, 2015).

Drugs or natural compounds with capacities to attenuate inflammatory signaling pathways associated with reactive gliosis should theoretically mitigate CNS inflammatory or glioma advanced processes. These are known to include specific targets such as janus kinase/signal transducer and activator of transcription (JAK/STAT) (Ben Haim, Carrillo-de Sauvage, 2015a, Ben Haim et al., 2015b, Wang et al., 2015b), nuclear Factor of Kappa light polypeptide gene enhancer in B-cells (NF-κB), calcineurin, Mitogen-Activated Protein Kinases (MAPKs) (Ben Haim, Carrillo-de Sauvage, 2015a) or Notch1-STAT3-ETB (LeComte et al., 2015). These are the indirect controls account for biological events such as induction of iNOS, release of NO, MCP-1 and CINCS. (Kesanakurti et al., 2009, Lindemann, Marschall, 2015) It is likely in the future, that nutraceuticals could be utilized to both attenuate risk of CNS inflammatory processes and age-related cognitive decline (Wang et al., 2015a) as well as survival, migration and invasion of glioblastomas (Lamy et al., 2015, Leidgens et al., 2015).

The findings in the work, establish preliminary evidence showing a number of anti-inflammatory natural products, effective in the glioma model. Some of these, as in the case of green tea (*Camellia sinensis*) or its biologically active polyphenol: EGCG (epigallocatechin 3-gallate) are supported by a plethora of existing studies describing protection against neuroimmunological related insults such as alpha synuclein (Siddique et al., 2014) Abeta (1–42) (Lee et al., 2009) experimental hydrocephalus (Catalao et al., 2014) ischemic stroke (Liu et al., 2013) and aluminum chloride neurotoxicity. (Jelenkovic et al., 2014) Likewise, the immune modulating effects of green tea component EGCG (epigallocatechin 3-gallate) can sensitize effects of temozolomide in models of intracranially implanted human U87 or U251 glioblastoma and enhance GBM to ionizing radiation (Chen et al., 2011, McLaughlin et al., 2006). Similar effects are often reported for turmeric root (*Curcuma Longa*)/curcumin both in attenuation of CNS injuries (Li et al., 2015a, Li et al., 2015b, Zhu et al., 2014) as well as comprising anti-cancer modulators in general and particularly for glioblastoma. (Dhandapani et al., 2007, Karmakar et al., 2007, Perry et al., 2010)

However, for other substances reported in the current work, there is meager research available. For example, while there are a few studies showing potential for Feverfew (*Tanacetum parthenium*) in the treatment of migraines (Tassorelli et al., 2005) or others of benefit in ischemic injury such as Ganthoda (*Valeriana wallichii*) (Rehni et al., 2007) Tansy (*Tanacetum vulgare*) and (Vafaee et al., 2012), Bay Leaf (*Laurus nobilis*) (Cho et al., 2010), clearly there is a need for investigation in to glial/glioma related neuro-inflammatory effects.

There is little to no research existing on therapeutic benefit of Maddar Root (*Rubia tinctoria*), or Red Sandlewood (*Pterocarpus santalinus*).

Like green tea and turmeric root, use of ashwaganda (*Withania somnifera*) is widely known to treat anxiety, depression and neurodegeneration (Ahmad et al., 2005, Baitharu et al., 2013, Bhattacharya et al., 2000, Jain et al., 2001), not only in research literature, but also historical documents and ancient medical texts pertaining to Ayurvedic medicine. Ashwaganda has beneficial effects on diseases of cardiovascular nature (Ravindran et al., 2015a), obesity, diabetes, infection, cancer (Choi and Kim, 2015, Khazal et al., 2014, Rai et al., 2016), arthritis (Gupta and Singh, 2014, Khan et al., 2015) and gastric inflammation (Kim et al., 2016). The major constituent in Ashwaganda, identified as Withaferin A, itself is an inhibitor of HMG-CoA, angiotensinogen-converting enzyme, (Ravindran et al., 2015b) can lower total cholesterol, triglycerides, higher HDL/LDL ratios (Shukla et al., 2014) and reduce severity or incidence of myocardial infarction, (Khalil et al., 2015) stroke (Ahmad et al., 2015, Raghavan and Shah, 2015, Sood et al., 2015) and hypertension. (Kaur et al., 2015) As far as glial cells, little has been investigated, but it has been reported that Withaferin A has potential in treatment of glioblastoma multiforme (Chang et al., 2016) with possible future therapies to target brain tumors (Kataria et al., 2015).

Elecampagne Root (*Inula helenium*) has also been referenced in ancient texts, referred to as elf-Dock or elfwort being used to treat digestion, nerve pain and asthma and having been sold in sweets, candies and cakes in London for aiding in respiratory health (Al-Gammal, 1998). Although future research will be required to evaluate the value of this herb for diverse neuro-inflammatory disorders, previous reports confirm its ability to attenuate inflammation in LPS-activated RAW264.7 cells where it activates p38 MAPK/Nrf2/HO-1 pathways also reducing inflammation in cecal ligation-induced sepsis in mice.(Park et al., 2013) Lastly, Ganthoda (*Valeriana wallichii*), is a well known sleeping aid (valerian) (Toolika et al., 2015) having historic reputations as a traditional Chinese medicine for treating insomnia, nervous disorders, epilepsy, and vision impairment. Neuroprotective activities has been demonstrated due to activity or iridoids (Zhang and Ding, 2015) which are reportedly capable of attenuating DAergic degeneration in MPTP treated mice (Sridharan et al., 2015) and cerebral injury from stroke.(Rehni, Pantlya, 2007) However, more research is needed to envelope diverse therapeutic values.

In summary, the data from this paper serve as a guide for future studies which may explore mechanism of action, constituents within and capabilities for defined natural products to prevent CNS inflammatory related degenerative injury and forces which drive aggressive malignant gliobastoma.

Acknowledgments

This research was supported by the National Institute of Minority Health and Health Disparities of the National Institutes of Health through Grant Number 8 G12MD007582-28 and Grant Number 1P20 MD006738-01.

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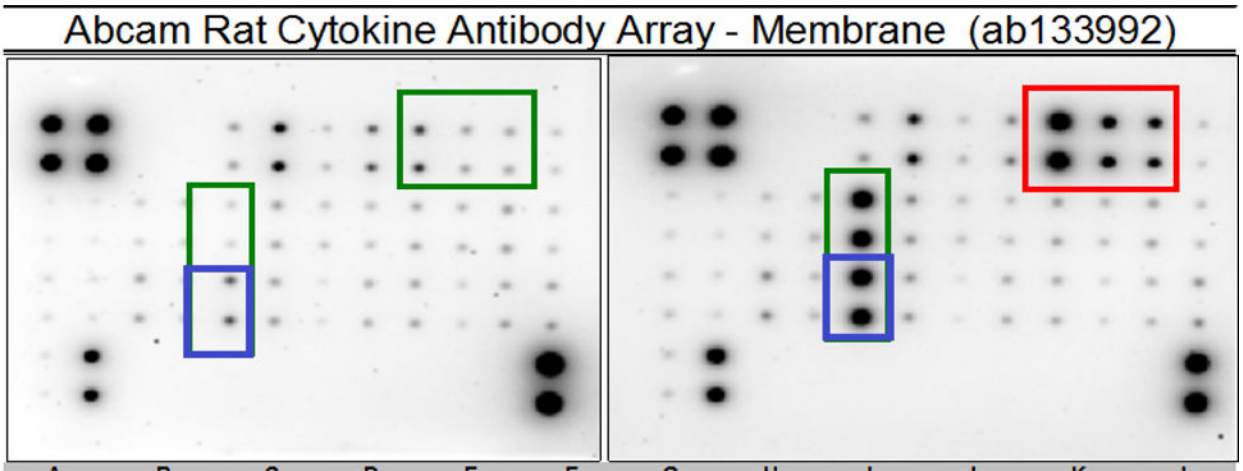
Highlights

- Central nervous system (CNS) inflammation involves pro-inflammatory processes within glial cells, which is often associated with neurodegenerative diseases.
- C6 glioma cells were evaluated for profile changes in pro-inflammatory molecules upon treatment with lipopolysaccharide (LPS)/Interferon gamma (IFNγ) by antibody array
- Differential shifts were evident for MCP-1, NO_2- and cytokine-induced neutrophil chemoattractants (CINC) 1, CINC 2a and CINC 3
- Over 1400 natural products were evaluated for capacity to reduce NO_2- in fully viable cells and only 1.77% showed anti-inflammatory effects comparable to steroidal and non-steroidal reference drugs
- Of these, many also demonstrated significant attenuation of CINC-3 determined by ELISA
- Lead products included : Ashwaganda (*Withania somnifera*), Elecampagne Root (*Inula helenium*), Feverfew (*Tanacetum parthenium*), Green Tea (*Camellia sinensis*), Turmeric Root (*Curcuma Longa*) Ganthoda (*Valeriana wallichii*), Tansy (*Tanacetum vulgare*), Maddar Root (*Rubia tinctoria*), Red Sandle wood (*Pterocarpus santalinus*), Bay Leaf (*Laurus nobilis*, Lauraceae), quercetin, cardamonin, fisetin, EGCG, biochanin A, galangin, apigenin and curcumin.

A



	A	B	C	D	E	F	G	H	I	J	K	L
1	Pos	Pos	Neg	Neg	Activin A	Agrin	B72/CD86	B-NGF	CINC-1	CINC-2α	CINC-3	CNTF
2	Pos	Pos	Neg	Neg	Activin A	Agrin	B72/CD86	B-NGF	CINC-1	CINC-2α	CINC-3	CNTF
3	Fas Ligand	Fracktaline	GM-CSF	ICAM-1	IFN-g	IL-1α	IL-1b	IL-1 R6	IL-2	IL-4	IL-6	IL-10
4	Fas Ligand	Fracktaline	GM-CSF	ICAM-1	IFN-g	IL-1α	IL-1b	IL-1 R6	IL-2	IL-4	IL-6	IL-10
5	IL-13	Leptin	UX	L-Selectin	MCP-1	MIP-3a	MMP-8	PDGF-AA	Prolactin R	RAGE	Thymus Chemokine-1	TIMP-1
6	IL-13	Leptin	UX	L-Selectin	MCP-1	MIP-3a	MMP-8	PDGF-AA	Prolactin R	RAGE	Thymus Chemokine-1	TIMP-1
7	TNF-α	VEGF	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Pos
8	TNF-α	VEGF	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Pos

B

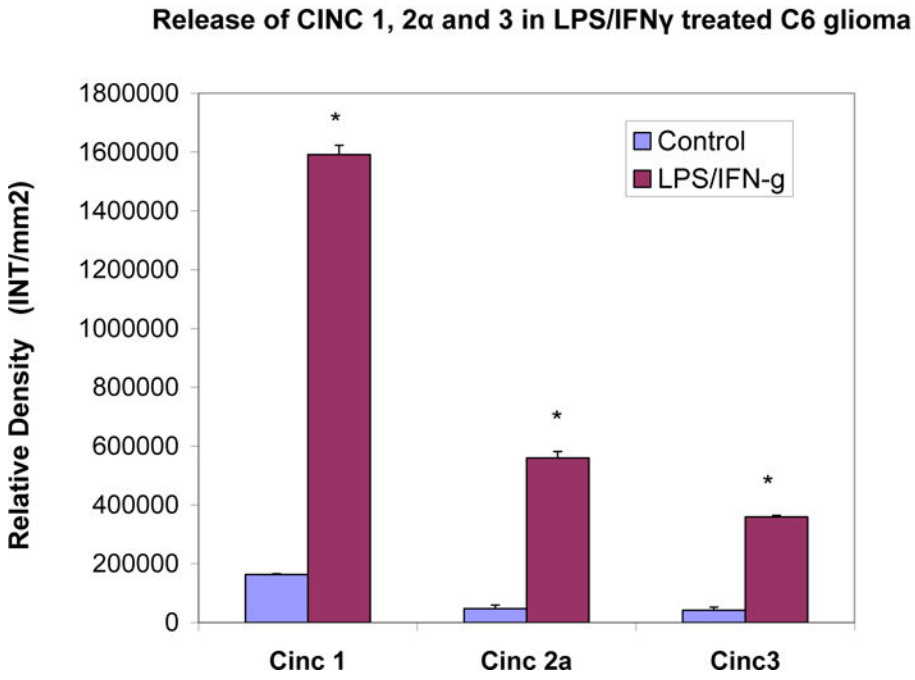


Figure 1A.

Cytokine release in LPS/IFNγ treated C6 glioma cells at 24h. The blot image (Top) and corresponding array grid layout (Bottom) are presented. **(B)** CINC1,2a and 3 release were significantly upregulated in LPS/IFNγ treated C6 glioma. The data represents relative

density and are expressed as the Mean ± S. E. M., n=4. Differences between resting and activated cells were determined using a Student's t-test, (*) P<.001.

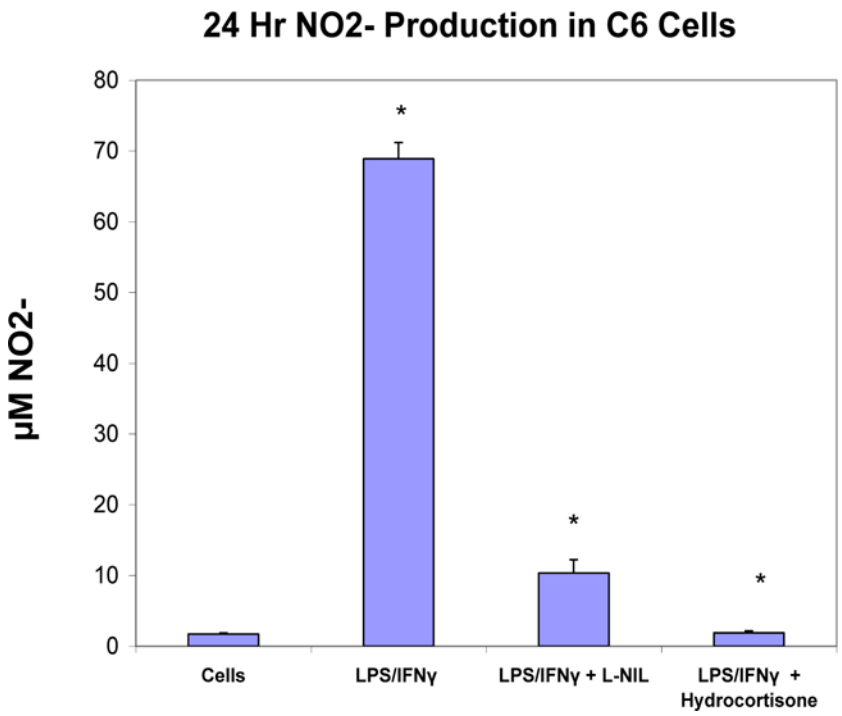


Figure 2A.

NO^{-}_{2} production in resting and LPS/IFNγ treated C6 glioma cells ± selective iNOS inhibitor: L-NIL (20μg/ml) or hydrocortisone (20μg/ml). The data represent NO2− produced (μM) and are expressed as the Mean ± S. E. M., n=4. Differences between resting and activated cells were determined by a Student's t-test (*) P<.001, and LPS vs. L-NIL and hydrocortisone treated cells [*].

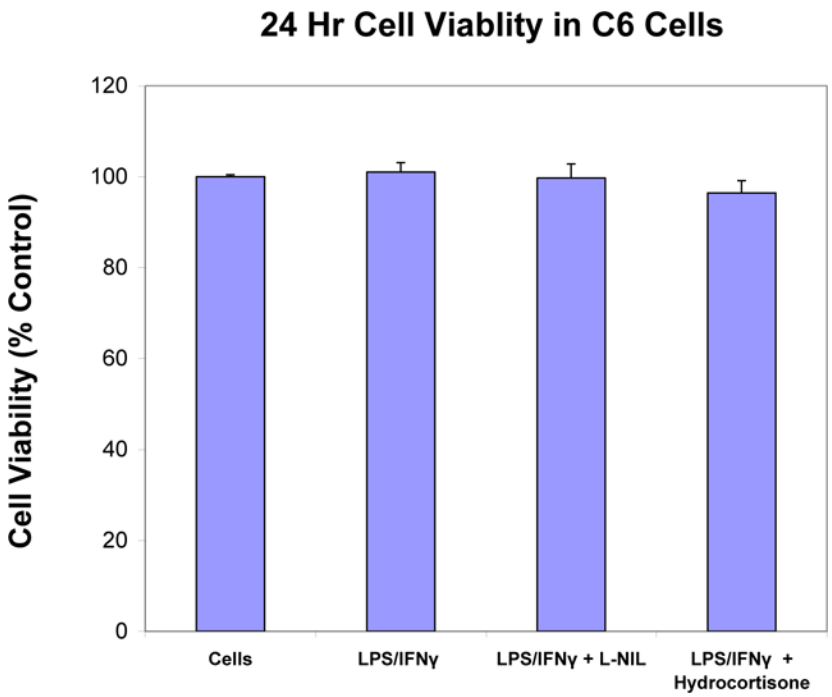


Figure 2B.

Cell viability in resting and LPS/IFNγ treated C6 glioma cells ± selective iNOS inhibitor: L-NIL (20μg/ml) and hydrocortisone (20μg/ml). The data represent cell viability as % control and are expressed as the Mean ± S. E. M., n=4. Differences between resting and activated cells were determined by a Student's t-test (*) P<.001, and LPS vs. L-NIL and hydrocortisone treated cells [*].

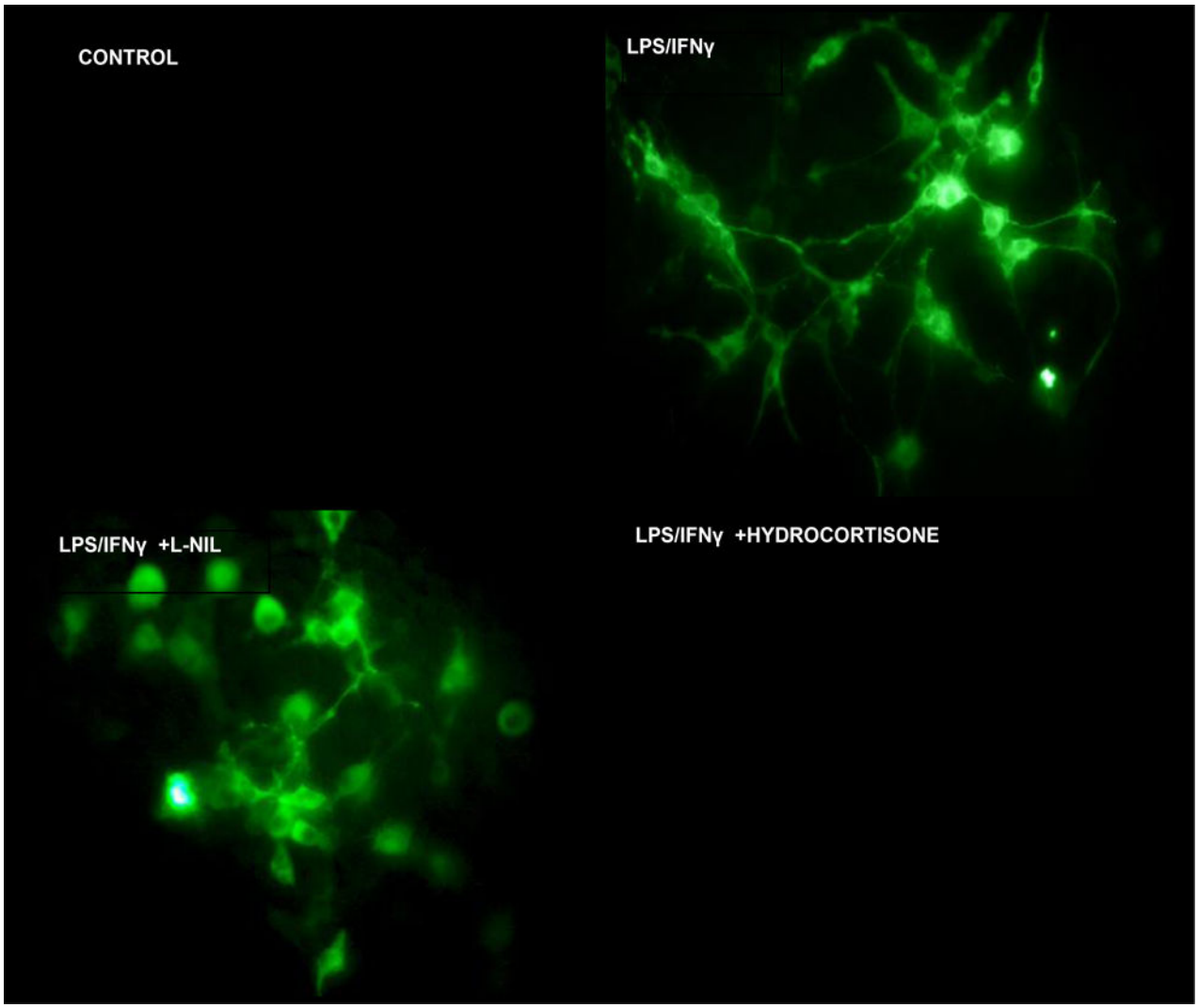


Figure 2C.

ICC imaging of iNOS expression using rabbit anti mouse iNOS/goat anti-rabbit Alexafluor 488, in fixed permeabilized: Controls: resting C6 cells, LPS/IFNγ treated, LPS/IFNγ treated + L-NIL (20μg/ml) and LPS/IFNγ + hydrocortisone (20μg/ml).

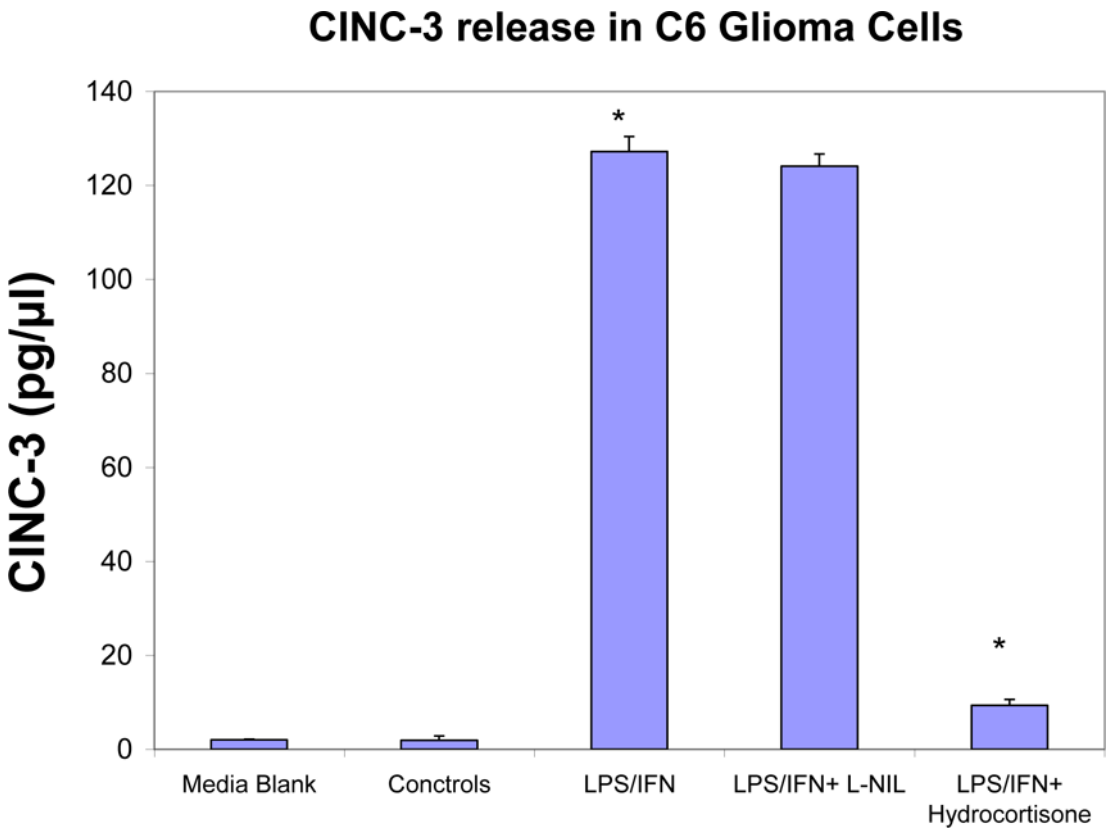


Figure 2D.

ELISA quantification of CINC-3 released in supernatant by LPS/IFNγ treated C6 glioma cells at 24 hours ± selective iNOS inhibitor: L-NIL (20µg/ml) and hydrocortisone (20µg/ml). The data represent CINC-3 (pg/µl) and expressed as the Mean ± S. E. M., n=3. Differences between resting and LPS/IFNγ activated cells were determined by a Student's t-test (*) P<. 001, and LPS/IFNγ vs. L-NIL and hydrocortisone treated cells (*) P<.001.

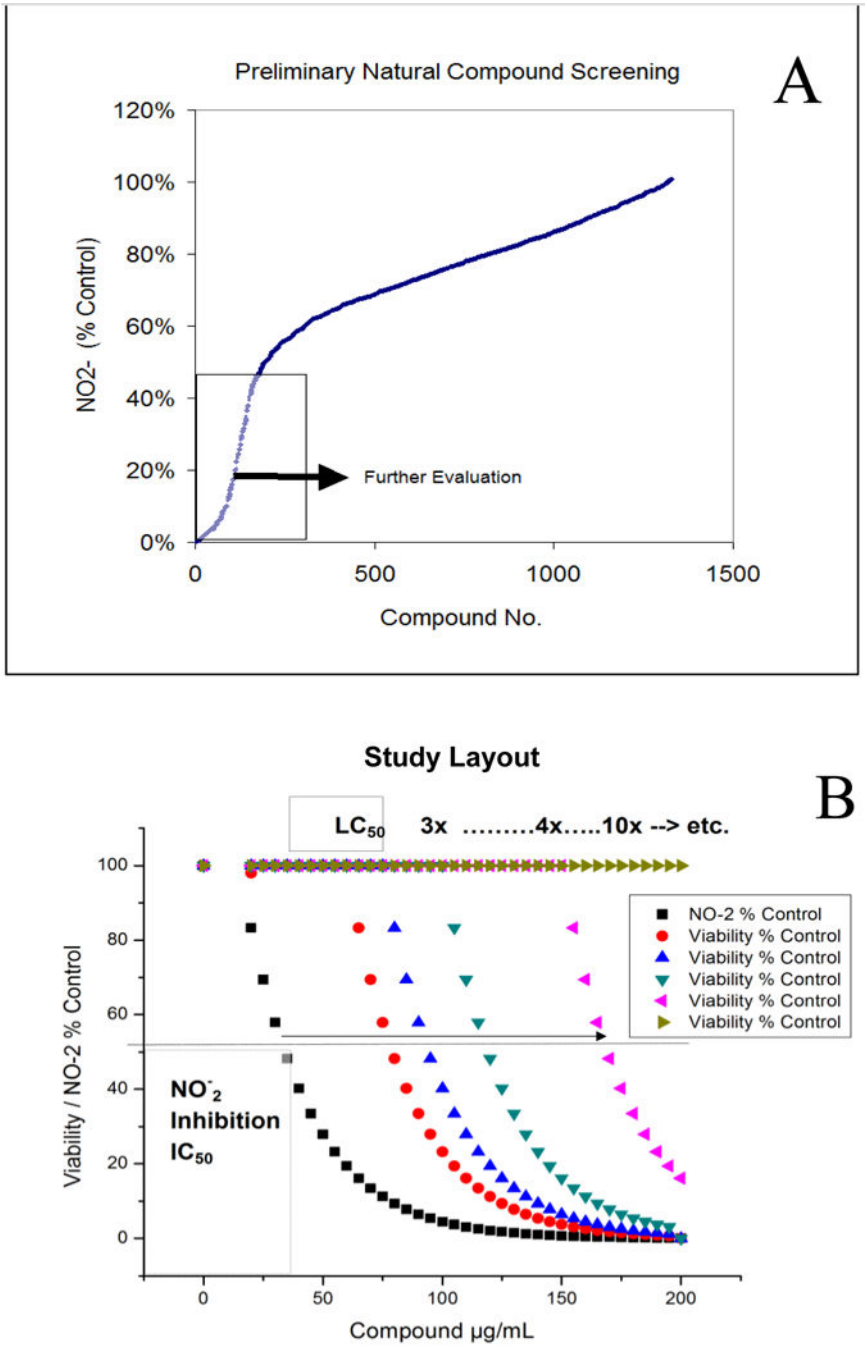


Figure 3.

[A] The basic study layout consisted of a primary Tier 1 elimination by which all herbs were tested to reduce LPS/IFNγ induced NO-2 in C6 cells, with maximum working concentrations : 230µg/mL (herbal extracts) and <92µg/ml (metabolites, drugs and polyphenolics). **[B]** Compounds displaying an IC_{50} below 1st Tier concentrations were further evaluated where toxicity/anti-inflammatory effects were evaluated simultaneously and an *in vitro* efficacy index *i*EI differential is established (LC_{50}/IC_{50}).

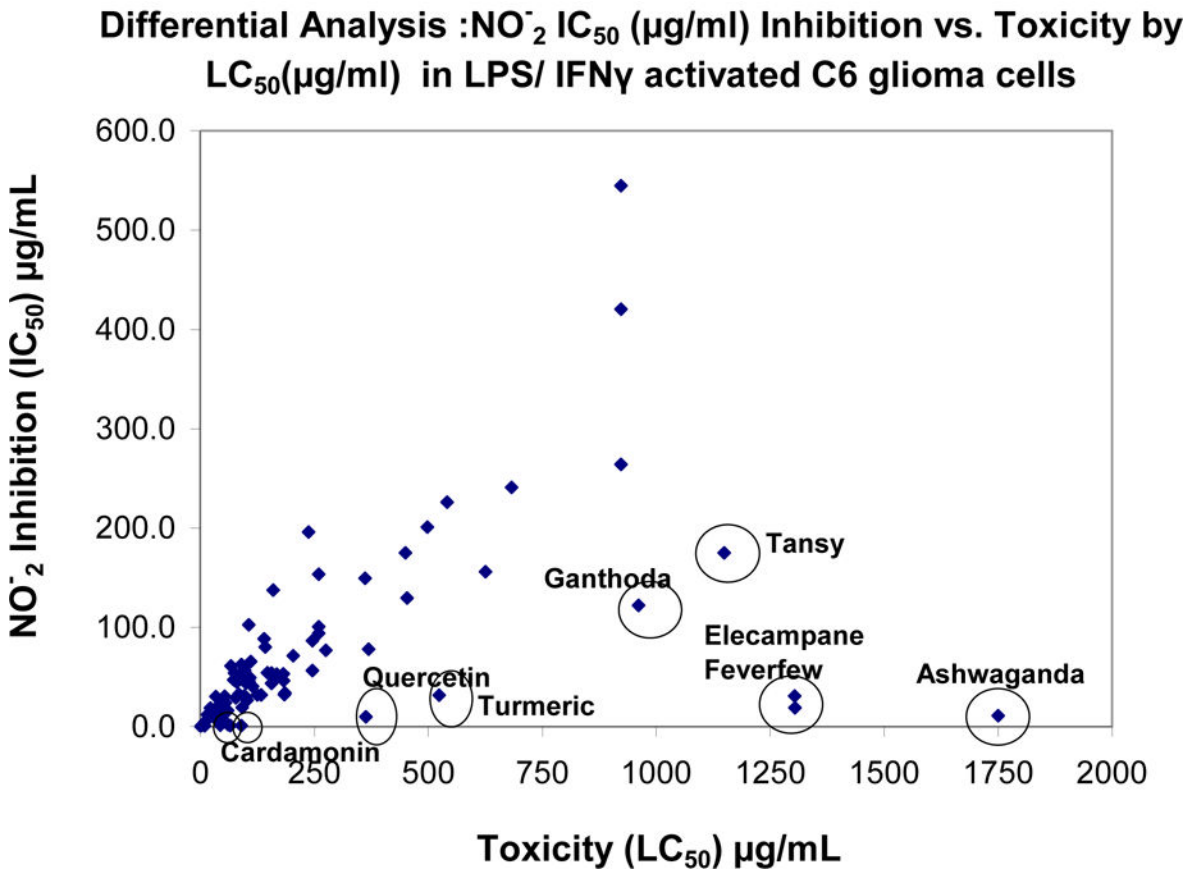
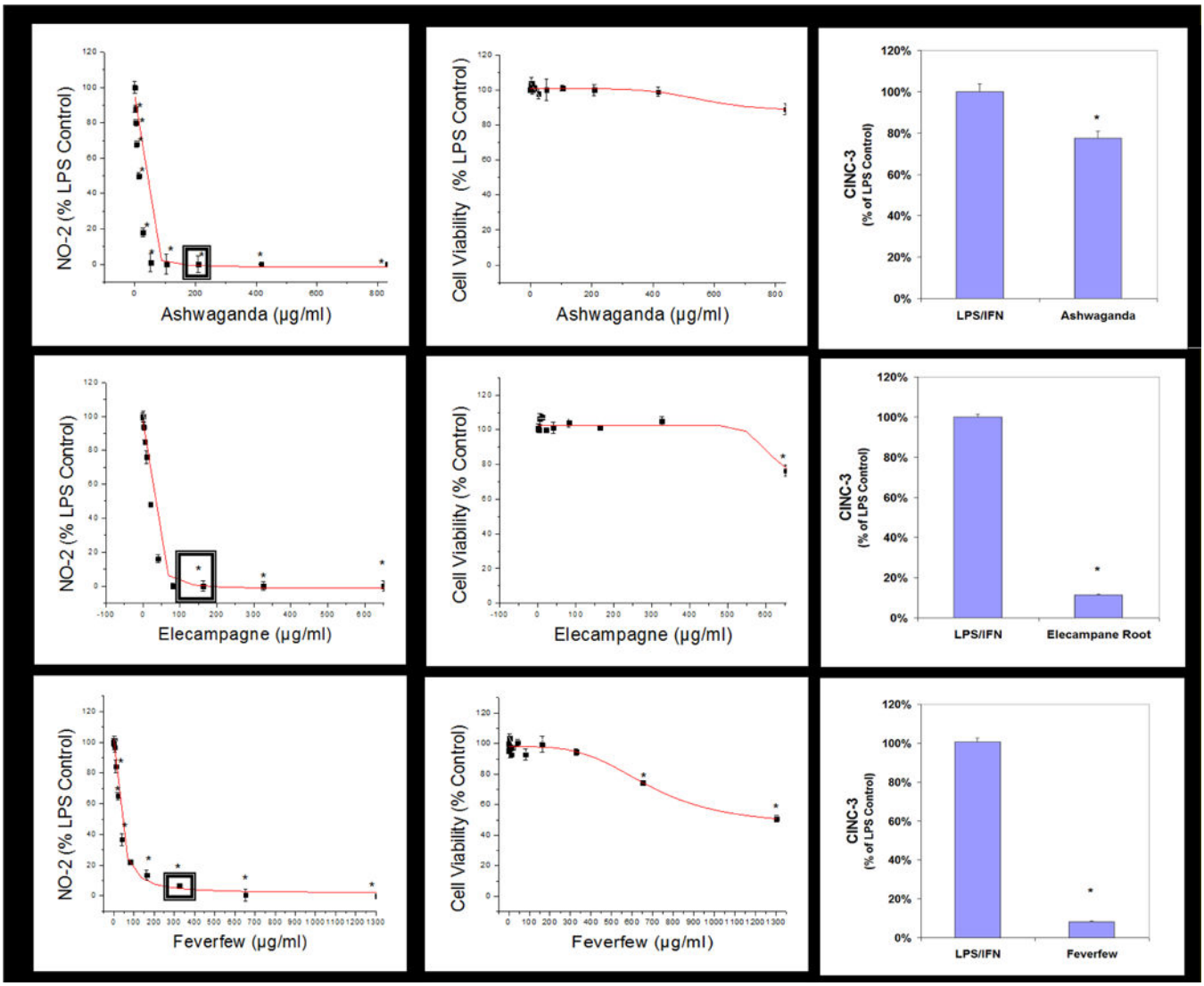


Figure 4.

Table 1 scatter – plot showing HTP Screening Results by LC_{50} (µg/ml) X- Axis and IC_{50} (µg/ml) Y-Axis.



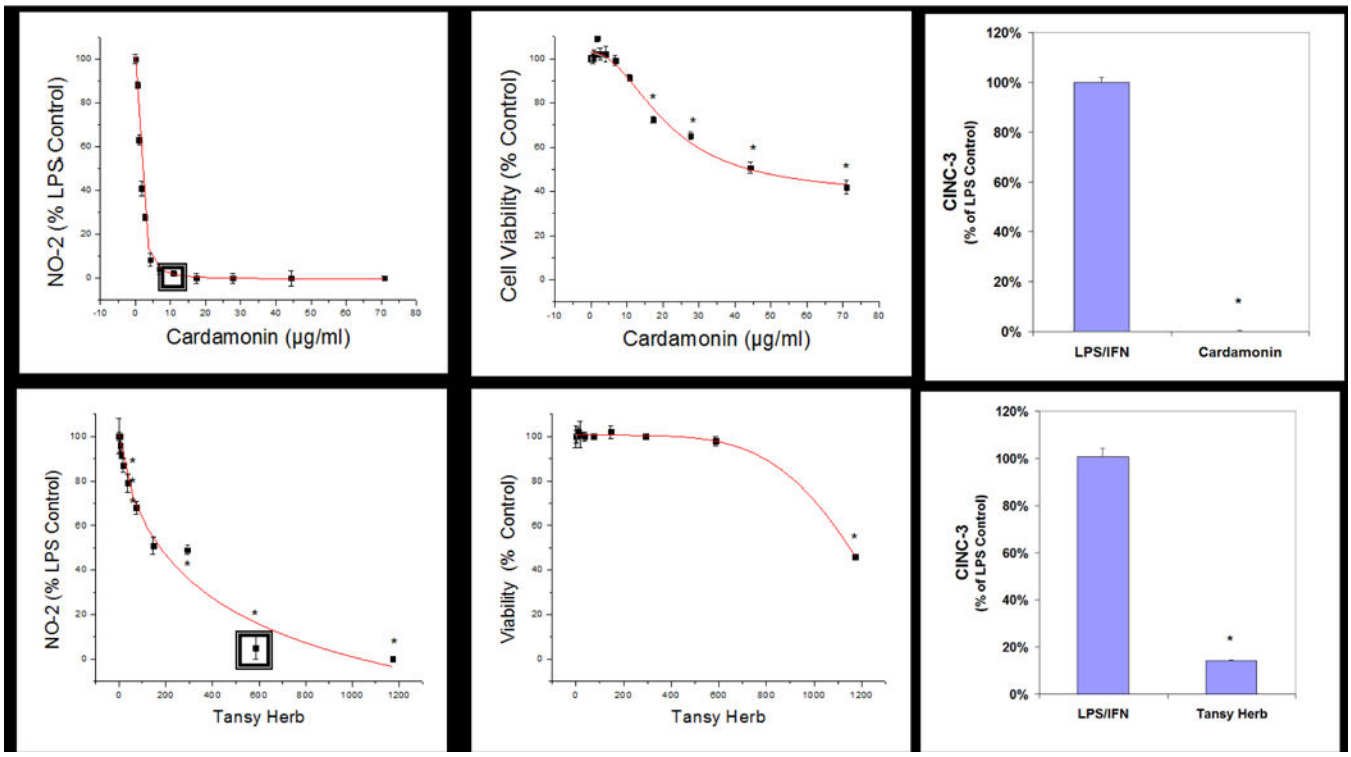


Figure 5.

Sample linear regression profiles for dose response used to calculate LC_{50s} (µg/ml) and IC_{50s} (µg/ml) where sub lethal-anti-inflammatory single concentrations were used to evaluate for CINC-3 release in LPS/IFNγ glioma demarcated by a (◘). The data represents cell viability and NO2− (% LPS/IFNγ Control), and significance from controls were evaluated with a one-way ANOVA, with a Tukey Post Hoc test, (*) P<.05. The data represents CINC-3 (as % LPS/IFNγ) and expressed as the Mean ± S.E.M., n=3. Differences determined by a Student's t-test (*) P<.05.

Table 1

Efficacy of natural anti-inflammatory products relative to toxicity in LPS/IFNγ activated C6 cells. The data represent LC_{50} values for toxicity and IC_{50} values for NO-2 reduction as determined by regression analysis, and *in vitro* efficacy index *i*EI differential is established as (LC_{50}/IC_{50}). < [Max concentration evaluated].

C6 Glioma : 3μg/mL LPS + 30ng/mL IFNγ						
Substance	***Botanical Name***	**IC_{50} (μg/mL)**		**LC_{50} (μg/ml)**		***i*EI**
Ashwaganda	***Withania somnifera***	**11.1**	**>**	**1750**	**>**	**150.1**
Hydrocortisone	***N/A***	**1.2**	**>**	**90**	**>**	**75.7**
Elecampane Root	***Inula Helenium***	**18.9**		**1304.5**		**69**
L-N-lysine dihydrochloride	***N/A***	**1.2**	**>**	**66**	**>**	**54.5**
Feverfew	***Tanacetum parthenium***	**31**		**1304**		**42.1**
Quercetin	***N/A***	**10**	**>**	**363.6**	**>**	**36.3**
Turmeric Root	*Curcuma Longa*	31.8		523.8		33
Dexamethasone	*N/A*	1.9	>	61.5	>	32.4
Cardamonin	*N/A*	1.5		43.9		29.9
Apicidin	*N/A*	0.8		9.8		12.3
Ganthoda	*N/A*	122.1	>	961.5	>	7.9
Tansy Herb	*Tanacetum vulgare*	175		1149		6.6
Diclofenac	*N/A*	32.3		184.5		5.7
Maddar root	*Rubia tinctorum*	34		186		5.5
Fisetin	*N/A*	10.1		48.4		4.8
EGCG	*N/A*	19.3	>	92	>	4.8
Biochanin A	*N/A*	78	>	369.2	>	4.7
Tolfenamic Acid	*N/A*	10.8		48		4.4
Galangin	*N/A*	56.5		246.1		4.4
Red Sandlewood	*Pterocarpus santalinus*	32		133.6		4.2
Apigenin	*N/A*	13.2		54.1		4.1
Bay Leaf	*Laurus nobilis*	156		626		4
Osha Root	*Ligusticum porteri*	46.4		183		3.9

C6 Glioma : 3μg/mL LPS + 30ng/mL IFNγ					
Substance	***Botanical Name***	**IC_{50} (μg/mL)**		**LC_{50} (μg/ml)**		**iEI**
Green Tea Std Sigma T5550	*Camellia sinensis*	32	>	125	>	3.9
Curcumin	*N/A*	26.8		102.8		3.8
Kalijiri Purple Fleablame	*Centratherum Anthelminticum*	16.2		60.2		3.7
Genistein	*N/A*	8.7		31.4		3.6
Glabridin	*N/A*	12.7		45.9		3.6
Ellagic Acid	*N/A*	43.6	>	156.7	>	3.6
Myrrh resin	*Commiphora myrrha*	76.8		275.3		3.6
Trifala	*Blend*	129.4		453.6		3.5
Amla	*Phyllanthus emblica*	264		923.1		3.5
Liquid Gold	*N/A*	53.2		182.3		3.4
Phloretin	*N/A*	16.7		56.3		3.4
Silymarin	*N/A*	30		99.6		3.3
White Sage	*Salvia apiana*	52.9		167.9		3.2
Gallic Acid	*N/A*	31		96.7		3.1
Butein	*N/A*	1.8		5.6		3.1
Gromwell Root	*Lithospermum erythrorhizon*	54.1		156.7		2.9
Yerba Santa Leaf	*Eriodictyon californicum*	71.3		203.9		2.9
Bergamottin	*N/A*	86.5	>	246.2	>	2.8
Sage leaf	*Salvia officinalis*	241		683.1		2.8
Chaparral	*Larrea tridentata*	40.6		114.7		2.8
Gamboic acid	*N/A*	0.3		0.9		2.8
Daidzein	*N/A*	94	>	260	>	2.8
Javentri	*Myristica fragrans*	54.2		147.6		2.7
Dragons Blood	*Dioscorea hypoglauca rhizome*	29		78.4		2.7
Silybinin	*N/A*	32.7		85		2.6
Ibuprofen	*N/A*	100.6	>	260	>	2.6
Rosemary Leaf	*Rosmarinus officinalis*	174.9		450.2		2.6
Centipeda Herb	*Centipeda cunninghamii*	200.9		498.3		2.5

C6 Glioma : 3μg/mL LPS + 30ng/mL IFNγ					
Substance	***Botanical Name***	**IC_{50} (μg/mL)**		**LC_{50} (μg/ml)**	**iEI**
Dong Ling Cao	*Rabdosia rubescens*	149.5		361.5	2.4
Herb de province	*Blend*	226.1		541.4	2.4
Resveratrol	*N/A*	45.9		107	2.3
Baicalein	*N/A*	24		55.2	2.3
Alkanet Root	*Batschia canescens*	43.9		100.6	2.3
Indomethacin	*N/A*	49.4		109.1	2.2
Cannabadiol	*N/A*	4.3		9.5	2.2
Clove Powder	*Syzygium aromaticum*	420.4	>	923.1	> 2.2