BIOLOGICALLY RELEVANT SECONDARY METABOLITES OF *VACCINIUM ULIGINOSUM*: BIOASSAY-DIRECTED NATURAL PRODUCTS IDENTIFICATION OF ANTI-NEUROINFLAMMATORY AGENTS IN THE ALASKA BOG BLUEBERRY

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Abstract

Dietary blueberry supplementation has demonstrated numerous health benefits including improved learning and memory in aging and neurodegenerative models, neuroprotection from ischemic events, anti-diabetic properties, and modulation of multiple inflammatory cascades. Despite previous research on antioxidant components prevalent in blueberries, no adequate explanation for a molecular mechanism for the benefits of blueberry supplementation has been proposed. *Vaccinium uliginosum*, the Alaska bog blueberry, possesses higher concentrations of antioxidant components than commercial varietals, and exhibits a greater oxygen radical scavenging capacity, making it an excellent candidate for the identification of biologically relevant secondary metabolites. An approach of bioassay-directed natural products identification was utilized to identify compounds in the Alaska bog blueberry responsible for the inhibition of both a magnesium-dependent neutral sphingomyelinase and NADPH oxidase in TNF-α-induced SH-SY5Y human neuroblastomas. Five relevant metabolites were identified: β-sitosterol (**1**), ursolic acid (**2**), 3-O-(4-hydroxyphenylcarboxylic acid) 4-O-(β-D-glucopyranosyl) gallic acid (**3**), malic acid (**4**), and 2,3-dihydroxybutane-1,2,3,4-tetracarboxylic acid (**5**). Neither compounds **3** or **5** had been previously described as a natural product in the literature. The identification of these compounds in the Alaska bog blueberry provides new explanations as to the benefits of blueberry consumption and offers new avenues of research for nutraceutical treatment of neuroinflammation.

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List of Abbreviations

Aβ	Amyloid beta protein
ABBX	Aqueous blueberry extract
ACh	Acetylcholine
AChE	Acetylcholinesterase
AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis
AMPK	AMP activated protein kinase
Ang II	Angiotensin 2
AP-1	Activator protein 1
ARE	Antioxidant response element
ATP	Adenosine triphosphate
BBA	Blueberric acid
BBX	Blueberry extract
BDNF	Bone-derived neurotrophic factor
CAPK	Ceramide activated protein kinase
CAPP	Ceramide activated protein phosphatase
CGD	Chronic granulomatous disease
$CHCl_3$	Chloroform
CNS	Central nervous system
COSY	Correlation spectroscopy
COX-2	Cyclooxygenase 2
CREB	cAMP response binding element
CYP	Cytochrome P450
Cyt b_{558}	Flavocytochrome b_{558}
DA	Dopamine
DCM	Dichloromethane
DEPT	Distortionless enhancement by polarization transfer

DMSO	Dimethyl sulfoxide
DPG	Dimeric catechuic acid glycoside
EDTA	Ethylenediamine tetracarboxylic acid
EGCG	Epigallocatechin-3-gallate
ERK	Extracellular signal regulated kinase
ESI	Electrospray ionization
FAD	flavin adenine dinucleotide
$FADH_2$	Reduced FAD
FAM	Factor associated with nSMase activation
FB	Fairbanks
FX	Fox
GABA	Gamma-aminobutyric acid
GC	Glucocorticoid
GLUT4	Glucose transporter 4
GR	Glutathione reductase
GSH	Glutathione
GST	Glutathione S-transferase
GSX	Glutathione peroxidase
GTP-ase	Guanosine triphospatase
H_2DCFDA	2',7'-dihydrodicholorfluorecein diactate
HDAC2	Histone deacetylase-2
HMBC	Heteronuclear multiple bond correlation
HO-1	Heme oxygenase 1
HRP	Horseradish peroxidase
HSP70	Heat shock protein 70
HSQC	Heteronuclear single quantum coherence
IGF-1	Insulin-like growth factor
Iκβ	Inhibitory kappa beta
IL-1β	Interlukin 1 beta

IL-6	Interlukin 6
JNK	c-Jun N-terminal kinase
LC-MS	Liquid chromatography coupled to mass spectroscopy
LD50	Minimum lethal dose
LDL	Low density lipoprotein
LM	Lake Minchumina
LPS	Lipopolysaccharide
LR	Lipid raft
LTP	Long term potentiation
MA	Malic acid
MAChR	Muscarinic acetylcholine receptor
MAPK	Mitogen activated protein kinase
MBCD	Methyl-β-Cyclodextrin
MDA	Malonyldialdehyde
MDD	Minimum defibrinogenating dose
MED	Minimum edema dose
MEK	MAPK-kinase
MeOH	Methanol
MEP	Methylerythritol 4-phosphate
MIF	Migration inhibitory factor
MPP^+	1-methyl-4-phenylpyridinium
MPTP	N-methy-4-phenyl-1,2,3,6-tetrahydropyridine
MS	Multiple sclerosis
MS	Mass spectroscopy
mTOR	Mammalian target of rapamycin
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
MVA	Mevalonic acid
NAC	N-acetylcystein
NADPH	Nicotinamide adenine dinucleotide phosphate

NE	Norepinepherine
NF-E2	Nuclear factor erythroid-derived 2
NF-κβ	Nuclear factor kappa beta
NMDA	N-methyl-D-aspartic acid
NMDAR	NMDA receptor
NMR	Nuclear magnetic resonance spectroscopy
NO	Nitric oxide
NOESY	Nuclear overhauser effect spectroscopy
NOS	Nitric oxide synthase
NOX	NADPH oxidase
NP_{BB}	Non-polar blueberry extract
Nrf2	NF-E2-related factor-2
nSMase	Neutral sphingomyelinase
OA	Oleanolic acid
OBBX	Organic blueberry extract
ORAC	Oxygen radical absorbance capacity
OS	Oxidative stress
PD	Parkinson's disease
PGE2	Prostaglandin E2
PI3K	Phosphoinositide-3-kinase
PKC	Protein kinase C
PLA_2	Phospholipase A_2
PMA	phorbol 12-ester 13-myristate
PO_{BB}	Polar blueberry extract
PTP	Protein tyrosine phosphatases
Q-TOF	Quadrapole- time of flight
ROS	Radical oxygen species
SBP	Systolic blood pressure
SHRSP	Spontaneously hypersensitive stroke prone rats

SOD	Superoxide dismutase
SS	β-sitosterol
T2DM	Type II diabetes mellitus
TGF	Transforming growth factor
TLC	Thin layer chromatography
TNF-α	Tumor necrosis factor alpha
TNF-R1	TNF receptor 1
TRAIL	Tumor necrosis factor related apoptosis ligand
UA	Ursolic acid

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Dedication

For my parents, Michael and Sandy, who passed to me the value of continuing education in you're your professional and personal lives. From you I learned to explore my curiousities, intelligently question, and to persist. I am forever grateful of your support for the unorthodox path I have followed to this point, and the support you continue to provide. For my wife, Crystal, who has provided me motivation, intensity, and empathy. You helped me find balance in my life and hold me up when I need it the most. I honestly don't think I could have finished without you. I love you.

1.0 Introduction

1.1 Relevance of *Vaccinium uliginosum*, the Alaskan bog blueberry

Blueberries contain high levels of anti-oxidative polyphenolic compounds and have demonstrated powerful anti-oxidative, anti-inflammatory, neuroprotective, and anti-carcinogenic properties in both *in vitro* and *in vivo* models. *Vaccinium uliginosum*, the Alaskan bog blueberry, possesses unusually high concentrations of polyphenolic compounds, especially catechins and anthocyanins (Latti et al. 2010), and increased radical scavenging capacity compared to other strains of blueberries. This is likely due to specific adaptations required to cope with the unique environmental conditions. It has also been shown that the further North bog blueberries are harvested, the higher the total anthocyanin content. Alaska blueberries harvested from three geographical regions demonstrated significant environmental influence of anthocyanidin and proanthocyanidin concentration and composition (Kellogg et al. 2009). The Alaska bog blueberry may also produce unique secondary metabolic defenses which function as protective molecules in animal models. Considering commercial cultivars of blueberries produce such intriguing results, the Alaska bog blueberry is a natural candidate for investigation in anti-inflammatory and anti-oxidative protection.

A significant body of evidence has been established for both the benefits of antioxidant and whole blueberry supplementation over a wide range of experimental models. Blueberry supplementation improves learning and memory in senescent mice, has shown promise as a therapeutic in neurodegenerative disorders, to reduce ischemic damage in stroke, to improve vascular elasticity in hypertension models, and to improve insulin sensitivity (Shukitt-Hale et al. 2005). Most of the effects attributed to whole berry supplementation cannot be ascribed to a direct modification of the cellular redox state due to the low concentrations of bioavailable berry components. Blueberries most likely exert protective effects via altering signaling pathways and transcriptional processes (Carmelina et al. 2002). However, little research has been done to directly identify active compounds in blueberries which are responsible for the effects of whole berries noted in

the literature. Increasing this body of knowledge will lead to nutraceutical, and possibly pharmaceutical, applications.

1.2 Bioassay directed fractionation for the identification of biologically relevant compounds in complex extracts

Most research relating antioxidant defense from plant extracts to a biochemical pathway or pathology follows one of two general protocols: 1) whole extracts are assessed for modulation of a specific biochemical pathway (Williams et al. 2008), or 2) individual, previously isolated, compounds of interest are assessed for modulation of a specific biochemical pathway (Mandel et al. 2004). The former approach is an excellent screening method for determining the level of interaction of a biological extract in pertinence to a particular pathway's modulation or regulation. The latter strategy is typically used when studying a compound which has already been determined to be worth further investigation based on previous studies on related biochemical pathways. The downside of screening a whole extract for activity is that, although initially necessary, it fails to answer the question, "What component(s) of the extract is specifically responsible for the demonstrated activity." Subsequent work with known compounds amounts to probing with educated guesses, and if successful simply confirms the compound's relevance. Neither approach identifies a compound specifically based on its activity, and neither technique broadens the knowledge of what secondary metabolites might be considered biologically relevant. Indeed, screening for only known compounds omits the possibility that unknowns can be identified.

For this study the problem was addressed via bioassay directed fractionation as a means of identifying components in Alaskan bog blueberries which could be attributed to having inhibitory effects for NADPH oxidase and magnesium dependent neutral sphingomyelinase in SH-SY5Y human neuroblastomas. While most studies of berry components involve steps which intentionally simplify the extract with acid or enzymatic hydrolysis, this approach was eschewed. Whole berries were lyphilized to prevent decomposition or modifications and extracted without hydrolysis. Multiple extraction

techniques were applied during early screenings to ensure comprehensive extracts were assessed for biological activities. Only after identifying fractions of interest were methods optimized for isolation. Most importantly, the fractionation and identification process was foremost directly guided by bioassay results. Each iteration of fractions was assayed for activity, and each subsequent technique was applied specifically to identify active components. Secondary considerations included solution complexity and the relative yield of the interesting targets.

Our investigations are beginning to bridge the gap in knowledge for the neutraceutical applications of blueberries. The Alaskan bog blueberry is more than a sum of polyphenolic compounds. The bioassay-guided identification of biologically relevant compounds in Alaskan bog blueberries will provide for new known compounds to be included in broad screening studies and specific, mechanistic, *in vitro* experiments.

1.3 Review of the literature: Whole berry supplementation and the relevance of NADPH oxidase and neutral shingomyelinase modulation

1.3.1 Blueberry supplementation and neuroprotection: Implications in learning and memory

1.3.1.1 Psychomotor performance and cognition improvements in the context of neuronal signaling and oxidative insult

Oxidative stress (OS) is gaining wide acceptance as a primary factor in the degradation of signaling and behavioral aptitudes observed during aging and neurodegenerative disease (Krause 2007). It has also been shown in both behavioral and biochemical studies that supplementation with whole blueberries or whole blueberry extracts can reduce these effects. Aging negatively affects multiple receptor systems linked to cognition and motor performance. Aging generally is associated with the decline of sensitivity for the adrenergic, dopaminergic, muscarinic, and opioid receptors which physically manifests as reduced psychological and physical capacities. Much of

these effects are attributable to the long-term effects of radical oxygen species (ROS) on proteins and lipids. The CNS is particularly sensitive to oxidative stress despite its relatively high consumption of oxygen. ROS in the CNS has been linked to an increased ratio of oxidized to active glutathione (GSH), increased membrane lipid peroxidation, and decreased glutamine synthesis.

Blueberry supplementation has been shown to ameliorate cognitive decline in senescent rodent models. An 8 month course of blueberry supplementation for Fischer 344 rats, beginning at 6 months of age, proved to be effective at reversing numerous neuronal and behavioral age related deficits (Joseph et al. 1999). Performance in psychomotor tests, including rod walking and accelerating rotarod, improved significantly in blueberry supplemented, aged rats. Cognitive testing assessed by Morris water maze performance showed both reduced latency to find the platform and a reduced distance traveled to the platform.

Blueberry supplemented, aged rats showed an order of magnitude improvement in oxo-enhanced K^+ evoked release of dopamine (DA) from striatal slices (Joseph et al. 2000). Additionally, the supplemented group demonstrated nearly twice the GTPase activity in striatal slices stimulated by carbachol compared to control rats. Ca^{2+} recovery was also evaluated in striatal synaptosomes. The addition of blueberries to the diet significantly improved Ca^{2+} recovery post H_2O_2 treatment, showing no significant difference from controls. Despite these notable improvements, no difference in GSH levels was observed, indicating that the effects may be produced by a mechanism other than anti-oxidative. Even more significantly, the improvements in psychomotor testing correlated to dopamine response and Ca^{2+} recovery.

F344 rats fed blueberry supplemented diets demonstrated improvements in age related declines in motor learning and β-adrenergic function (Bickford et al. 2000). Considering that β-adrenergic function is implicated in motor learning, norepinepherine (NE) was used to inhibit Purkinje neurons via the potentiation of GABA. GABAnergic inhibition occurred in 75% of the recorded neurons in young rats but only in 30% in aged rats. Dietary supplementation of blueberries in aged rats dramatically improved

inhibition following treatment with NE, demonstrating levels near those of young rats. This corroborated earlier work on antioxidant enriched diets which demonstrated improved β-adrenergic function as well as decreased expression of mRNA for the inflammatory cytokine TNF-α in aged rats (Carmelina et al. 2002).

NMDA receptor-mediated long term potentiation (LTP) is generally considered a necessary component of memory formation. Age related cognitive decline is strongly associated with both the decrease in NMDAR-potentiated LTP and decreased expression of NMDAR subunits. Aged Fischer rats fed a normal diet exhibited 69% the LTP of young rats; however, an 8 week protocol of aqueous blueberry extract supplementation increased the strength of the aged rats' LTP to levels equivalent to young rats' (Coultrap et al. 2008). Levels of NMDAR subunit expression were not increased due to the supplementation protocol, but phosphorylation of the NMDAR was significantly increased, overcoming the deficit in NMDAR levels in aged rats.

Blueberry supplemented Fischer rats have an increase in activation of hippocampal heat shock protein 70 (HSP70) stimulated by lipopolysaccharide (LPS) and dopamine release (Shukitt-Hale et al. 2005). This shows an improvement in anti-inflammatory responsiveness and has been linked to protection against ischemia and ROS. The improvement in HSP70 response coincided with previously recognized increases in motor skill performance. Interestingly, rats supplemented with other berry species, which yielded higher concentrations of total anthocyanins, did not demonstrate the same degree of improvement in DA release or HSP70 activation. This suggests that either the anthocyanins are not the relevant component, or that the blueberries contain unique variants within the class which are more potent, or combinations which act synergistically.

Older adult men and women exhibiting aquired memory decline demonstrated significant improvement in paired associate learning and word list recall following a 12 week course of dietary wild blueberry juice supplementation (Krikorian, 2010). The subjects were fed approximately 7.5 ml juice / Kg bodyweight for the duration of the

study. Subjects also demonstrated reduced symptoms of depression and reduced blood glucose levels. The reduction in blood glucose is significant because insulinemia and diabetes have been shown to significantly increase the risk of dementia and AD. These results suggest that dietary consumtion of blueberry juice may decrease risk factors for the onset of dementia as well as improve cognitive function.

1.3.1.2 The presence of blueberry anthocyanins and polyphenols in neuronal tissue

Aged F344 rats fed a 2% diet of blueberry extract for an 8 week period have shown quantifiable levels of multiple unmodified anthocyanins in neuronal tissue (Andres-Lacueva et al. 2005). LC-MS analyses showed the presence of cyanidin-3-O-B-galactoside, cyanidin-3-O-B-glucoside, cyanidin-3-O-B-arabinose, malvidin-3-O-B-galactoside, malvidin-3-O-B-glucoside, malvidin-3-O-B-arabinose, peonidin-3-O-B-arabinose, and delphinidin-3-O-B-galactoside in the brains of blueberry supplemented rats. This demonstrates the ability of anthocyanins to cross the blood brain barrier and localize in regions of the brain associated with learning and memory. It appears the cation in anthocyanins causes increased resistance to modification by formation of aglycones, glucuronides or sulphates. Additionally, other flavonoids, including epicatechin and naringenin have been shown to localize in brain tissue following oral administration. These results lend credibility to *in vitro* studies of whole blueberry extracts as well as phenolic compounds, and although do not prove causation, lend more credibility to the conclusion that anthocyanins are involved in neuroprotection.

Although there is an increasing body of evidence for benefits pertaining to whole berry supplementation, similar results have not been achieved via the administration of single antioxidant compounds. Additionally, most studies which demonstrate blueberry mediated neuromodulation rarely confirm a whole body improvement in ROS scavenging capacity. This suggests that the results are not caused by total antioxidant levels in the fruit, but rather may be due to a specific combination of antioxidant/anti-inflammatory agents. Blueberries contain high levels of anthocyanin glycosides including malvidin, petunidin, peonidin, and cyaniding. Despite these compounds being found intact in the

CNS and the body of evidence for their anti-inflammatory and anti-oxidant actions, anthocyanins may not be solely responsible for the neurological and behavioral improvements shown in blueberry supplemented aged rats.

1.3.1.3 Modulation of IGF-1 and activation of MAPK signaling cascades, CREB, and BDNF

Insulin like growth factor (IGF-1) is a known regulator of the extracellular receptor kinase pathway, a modulator of neurogenesis in the hippocampus, and is therefore a significant factor regulating learning and memory formation. It has been demonstrated that IGF-1, along with other growth factors influence the rate of neurogenesis in the hippocampus. IGF-1 also affects established populations of neurons via activation of signaling cascades such as the mitogen activated protein kinases (MAPK), which in turn modulate extracellular signal regulated kinase (ERK 1/2) and the subsequent activation of cAMP response binding element (CREB). MAPK response is critical to the formation of short term memory in the hippocampus and long term memory via downstream transcriptional regulation.

Short term blueberry supplementation in aged rats increased the proliferation of cells in the hippocampus while simultaneously demonstrating increased levels of IGF-1 and greater ERK 1/2 activation (Casadesus et al. 2004). The improved proliferation was positively correlated to spatial memory performance and a reduced number of memory errors in radial arm water maze testing. The increase in ERK 1/2 activation was also correlated to the improvements in memory. No association could be drawn between the increased levels of IGF-1 and proliferation. It is possible that dietary supplementation of blueberries affects the formation of short term memory via the IGF-1 up-regulation of ERK 1/2, while simultaneously affecting proliferation through another pathway such as c-Jun N-terminal kinase (JNK) and p38.

The activation of cAMP response element binding protein (CREB) is strongly associated with the formation of long term memory and plasticity, acting as a point of convergence for multiple signaling pathways including cAMP dependent kinase A,

calcium–calmodulin kinases, protein kinase C, and MAPK (Figure 1). CREB also regulates brain derived neurotrophic factor (BDNF) which has been shown to affect neuronal survival and memory in the central nervous system via local control of protein translation. A 12 week dietary supplementation (2%) of blueberries in both young and aged rats showed discrepancies between the two groups' spatial working memories by cross maze apparatus, demonstrated improvement in aged rats' spatial learning from 3 weeks on, and correlated the discrepancies and improvements to modulation of CREB and BDNF (Williams et al. 2008).

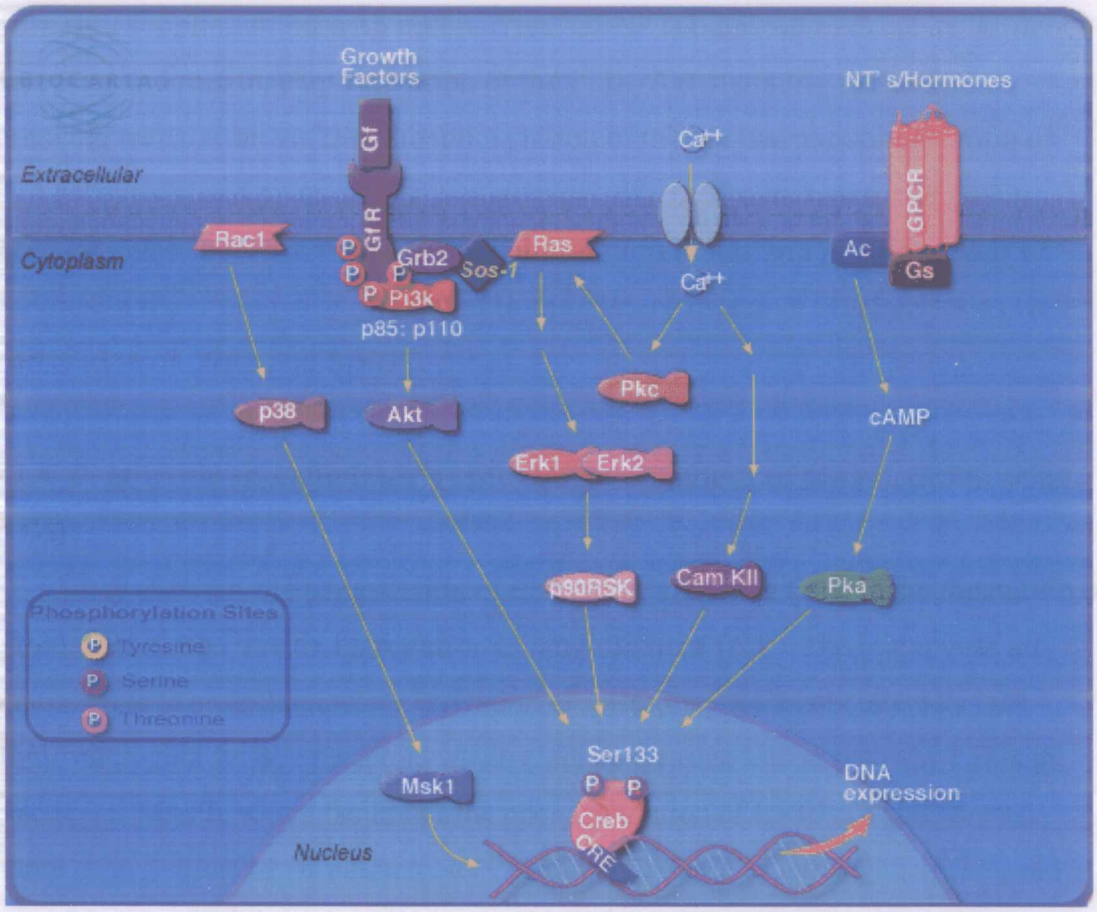


Figure 1 Transcription factor CREB and its extracellular signals. IGF-1 influences CREB activation via the PI3K/Akt pathway. Ras activation leads to the phosphorylation of RAF1, MEK, and ERK, leading to CREB activation. Additionally,

Rac1-mediated p38 phosphorylation and cAMP activation also influence CREB function. (Original NCI CGAP BioCarta Pathway)

Blueberry supplementation significantly increased hippocampal CREB phosphorylation. The activation of CREB was accompanied by the phosphorylation of ERK1/2, which is in agreement with findings which demonstrate flavanoids influence on protein and lipid kinases, especially the MAPK pathway. No effect was demonstrated on calcium calmodulin kinase or protein kinase A, which indicates that the supplementation protocol influenced CREB phosphorylation via ERK1/2 activation. Hippocampal pro-BDNF and mature BDNF levels also increased in mature rats supplemented with blueberries. BDNF is believed to exert its influence on learning and memory by activation of the PI3K/Akt signaling pathway, influencing translation via the phosphorylation of mTOR, the phosphorylation of NMDA receptors, and promoting a pro-survival effect. The increase in BDNF in treated rats was shown to result in phosphorylation of Akt and mTOR. Levels of flavanoids in the brain were correlated to the dietary intake of blueberries as well as the activation of CREB and BDNF.

1.3.1.4 Protection in Alzheimer's models: Involvement of MAPK, CREB, AChE, and GSH

A key event in the progression of Alzheimer's disease (AD) is the formation of amyloid oligomeres due to aggregation of amyloid beta (Aβ). The oligomeres are neurotoxic due to proinflammatory activation of microglia. Aβ likely interacts with multiple microglial surface receptors which mediate tyrosine kinase pathway. This in turn activates MAPK which facilitates the phosphorylation of CREB. These events promote an up-regulation of inflammatory cytokines, release of intracellular Ca^{2+}, and increased levels of ROS due to activation of NADPH oxidase (NOX), all of which can contribute to cell death. It has been shown that the activation of MEK1/2 and p44/42 (ERK 1/2) MAPK are involved in the NF-κβ-mediated release of the proinflammatory cytokines TNF-α and IL-6 in microglia when stimulated by LPS. In LPS-treated

microglia blueberry extract significantly reduced the phosphorylation of the p44/42 module (Zhu et al. 2008). This resulted in increased clearance of Aβ by microglia, inhibited the aggregation of Aβ into oligomeres, and suppressed microglial activation.

Blueberry treatments improved pMAPK signaling, reduced pCREB and PKC activation that had been altered by treatment with dopamine (Joseph et al. 2007). Blueberry pretreatment showed similar effects on Aβ (42) treated cells, excluding modulation of MAPK. This demonstrates that although blueberries and phenolic compounds have been shown to alter cellular signaling in cells under redox or inflammatory stress, the specific modulation is dependent on the stressor. Flavanoids have been shown to both activate and inhibit activation of MAPK transcriptional systems in a number of different models, so it is not unreasonable that the specific insult as well as the cellular environment determine the specific modulatory effects of blueberries.

The involvement of ROS signaling through MAPK and CREB is implicated in the relationship between age, neurodegeneration and memory formation. Primary hippocampal neurons of multiple ages were cultured and treated with Aβ in conjunction with blueberry extracts, and levels of pERK, pCREB, ROS, and GSH were evaluated (Brewer et al. 2009). Treatment of old, primary neurons with blueberry extract in conjunction with Aβ demonstrated reduced levels of pERK and pCREB compared to Aβ treatment alone. Simultaneous determination of ROS and GSH showed a pro-oxidant pulse from 10 to 30 minutes post treatment with blueberries alone. Treatment with Aβ showed a similar, but more prolonged increase in ROS, but pretreatment with blueberries followed by treatment with Aβ showed nearly no increase in ROS. Concomitant determination of GSH demonstrated that cells treated with blueberries had higher levels of GSH, and cells treated with both blueberries and Aβ had lower ROS and higher GSH than cells treated with Aβ alone. These results suggest that neuronal protection against Aβ toxicity is via a mechanism involving increased pools of GSH due to a cellular preconditioning.

It is possible that the blueberry extract acts as a pro-oxidant stressor which induces enzymatic up-regulation of GSH production, making the cell more fit to cope

with more intense oxidative insults. The increased pool of GSH would lead to a gross anti-oxidantive effect, and possibly explain the significant anti-oxidative properties observed in cells containing relatively low levels of polyphenolic compounds. It is also important to note that the short duration of the pro-oxidative burst would help prevent the cell from engaging in pro-apoptotic processes.

ERK 1/2 is essential for neuroprotection from ROS and inflammation as well as memory formation. Multiple flavanoids have demonstrated the capacity to increase Nrf2 expression, which leads to increased levels of GSH via the increased expression of glutamate–cysteine ligase. This effectively reduces the rate limiting step for GSH synthesis. The signaling cascade responsible for the activation of Nrf2 likely depends on ERK signaling.

Aged APP + PS1 transgenic mice, used as a model for Alzheimer's disease, fed a blueberry supplemented diet demonstrated no decline in cognitive performance via Y-maze testing compared to young mice. This was observed despite the fact that the total Aβ load in the aged mice was unchanged (Joseph et al. 2003). The improvements in memory versus untreated mice was likely due to improvements in ERK signaling and modifications to neutral sphingomyelin-specific phospholipase C activity.

Decreased muscarinic receptor (MAChR) sensitivity is associated with both aging and Alzheimer's disease, and this decrease is believed to be linked to declines in G-protein signal transduction. MAChR is also particularly sensitive to OS, and demonstrates increasing sensitivity with age which is linked to decreased neuronal function via Aβ management (Joseph et al. 2004). Influence of the cholinergic system on cognitive function is evident by the loss of cholinergic neurons exhibited in Alzheimer's patients. Cognitive deficits have been addressed therapeutically by agonizing cholinergic receptors or inhibition of acetylcholinesterase (AChE), increasing levels of available ACh. A 7 day course of blueberry extract administration via intraperitoneal injection (60 mg/Kg) increased cognitive performance in a passive avoidance test, decreased AChE activity in the brain and reduced lipid peroxidation (Papandreou et al. 2009).

1.3.1.5 Effects on inflammatory cytokines and ceramides

Aged F344 rats fed a 2% blueberry supplemented diet for 4 months exhibited marked decreases in TNF-α across multiple regions of the brain compared to controls (Goyarzu et al. 2004). These decreased levels of the inflammatory cytokine correlated with improvements in object recognition memory.

Levels of free ceramide increase in both regular aging and Alzheimer's disease. The cellular response to both Aβ and DA is exacerbated by ceramide. The presence of ceramide doesn't significantly alter the cell's ability to buffer Ca^{2+}, but does cause an increase in the time for a cell to depolarize when treated with oxotremorine. Treatment of the M1 transfected COS-7 cells with blueberries prior to insult mitigated the effects of ceramide (Ielinski and Fisher 2008).

TNF-α orchestrates inflammatory cascades linked to numerous neurological pathologies, traumatic and ischemic injuries, and neurodegeneration associated with both aging and disease. Increased levels of TNF-α can lead to MAPK activation, causing a cascade of phosphorylation events, which ultimately lead to increased ROS via NOX, activation of caspases, and the release of free ceramide lipid messengers via nSMase activation (Figure 2). Aqueous and organic extracts of wild Alaskan bog blueberries were shown to inhibit Mg^{2+} dependent nSMase in SH-SY5Y human neuroblastomas when treated with TNF-α (Gustafson et al. 2007). The inhibition was not due to a direct interaction or to bulk anti-oxidative effects. The inhibition was likely caused by upstream effects in the regulation of the signaling cascade or transcription. The specific effects of TNF-α largely depends on the cellular environment and other interacting factors (Manu and Kuttan 2008).

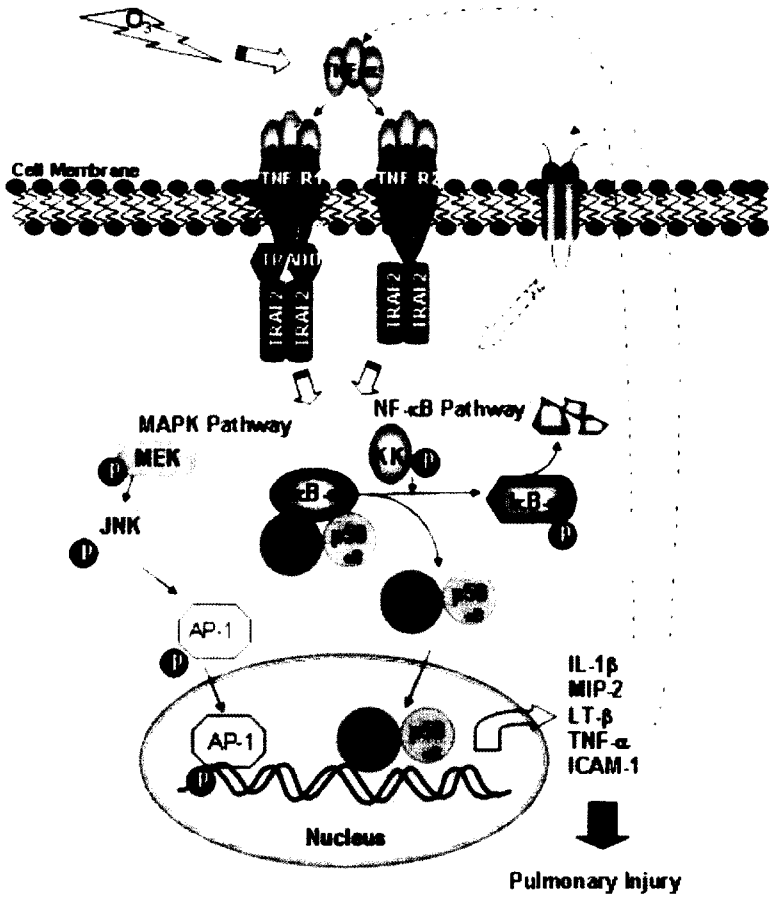


Figure 2 The regulation of MAPKs and NF-κβ via activation of TNF-Rs. Redox stress can lead to elevated levels of TNF-α. Activation of the TNF-Rs via binding of TNF-α leads to the phosphorylation of MEK, JNK, and AP-1 which leads to increased transcription of pro-inflammatory factors. Activation of the TNF-Rs also leads to the activation of the transcription factor NF-κβ (Cho et al., Am J Respir Crit Care Med, 2007).

NF-κβ is a common downstream target of CREB signaling. NF- κβ is responsible for the transactivation of multiple genes associated with inflammation including TNF-α, COX-2, IL-1β and inducible NO synthase (Figure 2). TNF-α then causes increased levels of NF- κβ, initiating a rapid inflammatory cycle. Treatment of neurons with blueberries may intervene in this cycle by decreasing CREB signaling.

1.3.2 Blueberry related neuroprotection in ischemia

Much of the damage caused by ischemic stroke can be attributed to oxidative stress caused by reactive oxygen species during reperfusion. Rats fed a diet containing 14.3% blueberries for only 6 weeks demonstrated a 57% reduction in neuronal loss in the hippocampal region following a hypoxia induced ischemic event (Sweeney et al. 2002). The CA1 and CA2 regions exhibited 66% and 68% less damage, while the CA3 region exhibited a 9% reduction. The hippocampus is an important region of the brain, critical to both learning and memory. Not only are blueberries high in antioxidant capacity, but they are very high in anthocyanins (25 times higher than strawberries or raspberries). Anthocyanins can cross the blood brain barrier in an unmodified, glycosylated state, and have been detected in the hippocampus of blueberry supplemented rats. It is plausible that the presence of anthocyanins can reduce the damaging effects of stroke by decreasing both platelet aggregation and the release of proinflammatory mediators.

Adult male Sprague-Dawley rats fed a blueberry enriched diet (2%) for 4 weeks exhibited significantly decreased infarction following induced ischemic stroke and reperfusion. Additionally, the ischemic hemisphere of blueberry-treated rats showed reduced caspase-3 activity, indicating a reduction in cytosolic cytochrome C induced apoptosis (Wang et al. 2005). The reduction in infarction correlated to improved post ischemic locomotor activities. Interestingly, pre-ischemic locomotor activities also showed improvement following only 4 weeks of supplementation.

Wild, low bush blueberry supplementation (3%) in spontaneously hypertensive stroke prone rats (SHRSPs) was shown to significantly reduce systolic blood pressure (SBP) and reduced levels of markers for renal stress. Factors contributing to hypertension include accumulation of oxidative damage due to high levels of reactive oxygen species produced primarily via NADPH oxidase activity. Additionally, NO is scavenged by superoxide, leading to endothelial disfunction and poor vasodilation. In the case of SHRSPs treated by blueberry supplementation, levels of urinary F2-isoprostanes did not indicate a significant decrease in systemic oxidative stress in the form of non-specific lipid peroxidation, which was significantly elevated in induced SHRSP

(Shaughnessy et al. 2009). Therefore the decrease in SBP was likely due to specific interactions regulating vasodilation, possibly via 8-iso-prostaglandin or a reduction in free superoxide.

1.3.3 Anti-diabetic properties of blueberries

The incidence of type II diabetes mellitus (T2DM) is increasing rapidly and at the global level. Native peoples, especially in arctic regions, are especially affected. Variables influencing the disease include lack of exercise, poor dietary habits, and genetic predispositions. Blueberries are believed to possess anti-diabetic properties, which should be reflected in cellular response to insulin. Peripheral cellular resistance to insulin is a primary symptom of T2DM. The anti-diabetic properties of the Canadian lowbush blueberry extracts were examined by a variety of methods (Martineau et al. 2006). Glucose transport in C2C12 skeletal myotubes and 3T3-L1 adipocytes was evaluated in response to a range of insulin treatments and incubation periods. Insulin secretion in β-TC-tet cells treated with glucose was determined. Proliferation of non-growth arrested, pancreatic β-TC-tet cells was also evaluated in the presence of berry extracts. Cellular protection against glucose toxicity was investigated by assessing cell viability for PC12-AC cells insulted with supra-physiological concentrations of glucose and treated with blueberry extracts. Extracts were prepared from the roots, stems, leaves, and fruit of the plant.

The fruit of the Canadian lowbush blueberry displayed strong anti-diabetic properties. Co-incubation of C2C12 myoblasts with blueberry and insulin showed significant improvement in glucose uptake as compared to insulin alone, but no significant changes were seen in the adipocyte model. This illustrates tissue specificity for the protection. Blueberry fruit dramatically stimulated β-cell proliferation (2.8 fold) versus non-treated cells. This has implications for late stage T2DM in which pancreatic β-cells rapidly degenerate. Lastly, berry fruit supplementation proved cytoprotective against glucose toxicity, improving cell viability by 20-30%.

The fermentation process modifies the phenolic content of blueberry juice and increases its anti-oxidant capacity. A six hour treatment of C2C12 myotubes and 3T3-L1 adipocytes with fermented Canadian lowbush blueberry juice showed a 48% and 142% increase in glucose uptake, respectively (Vuong et al. 2007). This was shown in both the presence and absence of insulin, indicating an insulin independent pathway. In the presence of insulin, muscle cells and adipocytes respond by translocating glucose transporter 4 (GLUT4) to the plasma membrane via insulin recptor activation. Muscle cells and adipocytes can also regulate glucose uptake independently of insulin via the activation of AMP activated protein kinase (AMPK), which can signal the translocation of GLUT4 in response to an increased ratio of AMP/ATP or rise in cytosolic calcium. Western blot analysis of blueberry treated C2C12 and 3T3-L1 cells showed a 1.9 and 3.2 fold increase in phosphorylation of AMPK versus controls, respectively.

Evidence for blueberries exhibiting hypoglycemic properties also includes *in vivo* models. Treatment of C57b1/6J diabetic mice with either polyphenol or anthocyanin enriched blueberry extracts significantly decreased symptoms of hypoglycemia (Grace et al. 2009). Labrasol, a commercial micro-emulsifier, used to increase the bioavailabilty of poorly absorbed oral drugs, was used to improve the absorption of the phenolic components. Both the phenolic and anthocyanin enriched extracts significantly lowered the blood glucose levels of mice treated with 500 mg extract per Kg body weight, by 33% and 51%, respectively. The discrepancy between the phenolic and anthocyanin enriched fractions suggests that the anthocyanin components are more significant as anti-diabetic agents. Pure anthocyanidins delphinidin-3-O-glucoside and malvidin-3-O-glucoside were both evaluated in conjunction with Labrasol, with malvidin-3-O-glucoside treatments showing significant reductions in hypoglycemia. Treatment of C57b1/6J diabetic mice with wild Alaskan blueberries from three geographical regions showed improved blood glucose regulation in both high fat and high carbohydrate diets following a 14 week supplementation (Kellogg et al. 2009). These results were obtained without Labrasol.

1.3.4 NADPH oxidase as a target for blueberry research

NADPH oxidase (NOX) is an assembly of protein subunits which collectively facilitate the transmembrane transfer of electrons via the generation of radical oxygen species (ROS) (Krause et al. 2007). NOX is involve in a multitude of physiological duties including, host defense and inflammatory response, regulation of gene expression, cellular signaling, and cell death. NOX misregulation is implicated in numerous pathologies, especially of the neuronal and vascular tissues. The fact that blueberry extracts demonstrate influence on ROS generation via non-antioxidative effects, makes NOX a particularly interesting target for studies pertaining to the identification of biologically relevant blueberry components.

The activation of NOX requires a series of specific protein/protein interactions. The phosphorylation of the p47 subunit catalyzes a series of organization steps which leads to the assembly and membrane localization of NOX and other subunits (Krause et al. 2007). Active NOX produces ROS in the form of superoxide which is initiated via an electron transfer between NADPH and FAD. The reduced $FADH_2$ transfers electrons to internal heme groups which then convert bound O_2 to superoxide. Superoxide is rapidly converted to hydrogen peroxide or can react with nitric oxide, forming peroxynitrite. Superoxide and nitric oxide have opposite influences on vascular rigidity, which may account for the positive effects blueberry supplementation has shown in hypertensive rat models.

NOX is clearly involved in host defense and inflammation (Krause et al. 2007). The respiratory burst initially associate with phagocyte killing is produced by NOX-mediated ROS generation. Individuals suffering from chronic granulomatous disease (CGD), who have poor NOX function, routinely exhibit serious infections. In addition to direct killing via hydrogen peroxide generation, NOX may also inactivate bacterial virulence factors. Although NOX is classically considered an inflammatory system, it also seems necessary for correct anti-inflammatory response. Low level production of

ROS is required for healthy cell function, including priming transcriptional networks which cope with cellular insults.

NOX can exert broad influence on cell function by the inhibition of phosphatases and activation of kinases. Redox sensitive cysteine residues in protein tyrosine phosphatase (PTP) activation sites are subject to modulation via oxidation. This mechanism provides a means for NOX to exert influence over numerous signal transduction pathways effecting differentiation, proliferation, and metabolism (Mrak and Griffin 2005). Additionally, NOX activity can lead to the activation of kinases including MAPK. The specific mechanism for this up-regulation is unknown, but it may be related to the inhibition of phosphatases. The MAPK family of signaling molecules has been associated with numerous studies concerning blueberry related improvements in learning and memory, neuroprotection, and inflammation.

NOX dependent generation of ROS is also involved in the regulation of inflammatory cytokine transcription. Expression of TNF-α, TGF-β1, and angiotensin are induced by elevated ROS. This upregulation is likely mediated by redox sensitive secondary messengers like the MAPKs. Other transcription factors, including NF- κβ and activator protein 1 (AP-1), which contain redox sensitive cysteine residues in their DNA binding domains, are also implicated in the induction of inflammatory cytokines. *In vitro* studies have shown reduced levels of inflammatory cytokines in blueberry treated systems, which may be due to MAPK mediated response to NOX regulation.

There is a large body of evidence for the link between NOX and cell death, but there is growing data indicating an equally important role in cell survival (Bissonnette et al. 2007). NOX is believed to cause cell death by non-specific modification of proteins, lipids, and DNA via ROS production. Sustained ROS stimulation of the redox sensitive MAPK enzymes JNK, ERK 1/2, and p38 can also act as pro-apoptotic signals. Conversely, anti-apoptotic signaling by ROS activated NF- κβ and Akt has been observed. It seems that the intensity and duration of the NOX activation, in conjunction with the global status of other influences on the redox sensitive, transcriptional targets all play a role in the interpretation of ROS as a cellular signal.

Elevated levels of ROS are evident in numerous pathologies, especially of the nervous and vascular systems. Elevated ROS is seen in senescence in normal aging, ischemic stroke reperfusion, and neurodegenerative disorders including Alzheimer disease and Parkinson's disease. NOX mediated ROS production can also acutely damage actin, causing a reorganization of the cytoskeletal structure (Gustafson 2008). Vascular hypertension and artherosclerosis also exhibit increased levels of ROS. Identifying neutraceutical interventions which may alleviate the onset, progression or symptoms of these diseases is of critical importance, especially in light of our aging population. It is clear that a more thorough examination of which blueberry-specific secondary metabolites modulate NOX function would benefit this task.

1.3.5 Neutral sphingomyelinase regulation of ceramide release as a target for blueberry research

Shingolipids are structural constituents of cellular membranes, and tend to be organized with cholesterols in lipid rafts. These domains also contain enzymes which specialize in the hydrolysis of sphingolipids which free messenger molecules into the cytoplasm. One such system is the hydrolysis of sphingomyelin by neutral sphingomyelinase (nSMase), which frees ceramide from the cell membrane (Clarke and Hannun 2006). Ceramides and ceramide metabolites, including ceramide-1-phosphate, glycosyl-ceramide, and sphingosine-1-phosphate, are important intracellular signaling molecules which influence inflammation, proliferative and apoptotic processes, neurodegeneration, and insulin sensitivity (Won et al. 2006). Ceramide regulation is influenced by ROS and the cellular redox environment. Depletion of GSH due to antioxidant defense has been shown to activate nSMase and increase levels of ceramide. Interestingly, ceramide can also directly influence the redox state of the cell by regulation of NOX (Nixon et al. 2009). These qualities make the nSMase an interesting target for evaluating the effects of blueberry supplementation and the identification of biologically relevant natural products in the Alaska bog blueberry.

Identified physiological stimuli activating nSMase activation include bioactive lipids, inflammatory cytokines, and oxidative stress (Won et al. 2006). Arachidonic acid, a key component in the production of prostaglandins via the COX-2 inflammatory response, has been shown to activate nSMase. The tumor necrosis factor receptor-1 (TNF-R1) has also been shown to regulate nSMase via the factor associated with nSMase activation (FAN). In the presence of TNF-α, TNF-R1 recruits FAN which activates nSMase. Redox stress also initiates nSMase activation, likely through the depletion of nSMase inhibitor, GSH.

While oxidative stress can initiate cellular signaling via the release of ceramide, ceramide and its metabolites can also influence the redox state of the cell by activation of NOX (Barth et al. 2009). Increased level of ceramide has been associated with increased NOX activation, and it has been suggested that this occurs through ceramide mediation of the GTPase, Rac. Ceramide is also responsible for the down regulation of the Bcl-2 genes which exert antioxidative effects by regulating cellular GSH levels (Clarke et al. 2006). Increased NOX activation and Bcl-2 down regulation would rapidly deplete cellular GSH. The decrease in pools of GSH would lead to a decreased cellular redox defense and subsequent increased activation of nSMase. Other factors in ceramide associated inflammatory signaling including increased expression of iNOS and NF- κβ, as well as enhanced clustering of TNF family receptors in lipid rafts.

Ceramides have numerous downstream targets including ceramide activated protein kinase (CAPK) and ceramide activated protein phosphatase (CAPP) (Arboleda et al. 2009). CAPK has been shown to inhibit Ras, which would decrease Raf phosphorylation and subsequent MAPK activation. CAPP belongs to the protein phosphatase A2 family which has been shown to be involved in caspase signaling and the down regulation of Bcl-2. Indirect targets influenced by ceramide also include PKC, PLA2, COX-2, and NF- κβ. Understanding the effects of Alaskan blueberry secondary metabolites of nSMase activation could provide new tools for combating inflammation.

Elevated levels of ceramides are associated with neurodegenerative disorders including Alzheimer's (AD) and Pakinson's disease (PD), as well as normal aging.

Levels of ceramide in PD patients showed a 13 fold increase over controls. Inhibition of nSMase ameliorated Aβ toxicity in AD models, indicating that ceramide is a key factor in neurdegeneration (Lee et al. 2004). Elevated levels of ceramide are likely an important factor in the early development of neurodegenerative diseases due to alterations in the neuronal survival, phosphatidil-inositol-3-kinase (PI3K)-AKT pathway, which leads to mitochondrial disfunction and apoptosis (Arboleda at al. 2009). The cellular PI3K-AKT response to growth factors reduces pro-apoptotic mediators while activating anti-apoptotic mediators. Increased levels of ceramide reduces cellular metabolism in the mitochondria, reducing ATP production by decreasing NADPH and GSH turnover. It is important to note that normal levels of ceramide are important in the effective recruitment and assembly of the tyrosine kinase receptor (TrkR) which is required for initiating regular PI3K response.

Alterations to the PI3K-AKT response to insulin and insulin-like growth factor-1 (IGF-1) via elevated levels of free ceramide may also be linked to insulin resistance. Increased levels of ceramide have been found in the skeletal muscle of obese, insulin resistant subjects versus lean, insulin sensitive subjects (Gill and Sattar 2009). Ceramide related insulin resistance in the brain has been shown to effect neuronal growth, plasticity, metabolism, Aβ protein processing, and gene expression. Additionally, reduced neuronal metabolism in Alzheimer's disease has been linked to insulin resistance, and manifests well before symptoms of cognitive disfunction (De la Monte 2009). There is also a link between type-2 diabetes and brain insulin resistance. Elevated levels of ceramide in the blood are free to cross the blood brain barrier, hence causing insulin resistance in the brains of type-2 diabetics. The regulation of nSMase activation via components of Alaskan bog blueberries may produce new insights into regulation of insulin resistance as well as early pathologies of neurodegeneration.

2.0 Methods

2.1 Evaluation of modulation of nSMase and NOX activation by Alaska bog blueberry components

Bioassays for inhibition of nSMase and NOX activation were performed as described in Appendices A-C by members of Dr. Kuhn's laboratory (University of Alaska Fairbanks): A) "Alaska Wild Blueberry Extracts Inhibit a Magnesium-Dependent Neutral Sphingomyelinase in Neuron Exposed to TNFα", B) "A Non-antioxidant Compound Present in Non-polar Blueberry Fraction Inhibits NADPH Oxidase-mediated Neuroinflammation", and C) "Ursolic Acid Isolated from Lipophilic Blueberry Fractions Inhibits NADPH Oxidase by Lipid Raft Modulation". These articles are included the supplementary materials as Appendices A, B, and C, respectively.

2.2 Materials

All solvents were purchased from VWR. Flash chromatography was performed with reagent grade solvents, and LC was performed with LC grade solvents. Flash chromatography was performed on 40 micron normal phase silica gel purchased from VWR. All authentic compounds used for NMR analysis and bioassay were purchased from Sigma. All other reagents and kits used for bioassays are described in Appendices A, B, and C.

2.3 Preparation of crude blueberry extract

Fruit of *Vaccinium uliginosum* were harvested in the interior region of Alaska when ripe and immediately frozen upon return to the laboratory. Whole berries were lyophilized until no change in mass was observed, crushed to powder and stored at -20^0 C until extracted. Crude extract was prepared by extracting 150 g blueberry powder with 500 ml aqueous acetone (70/30 acteone to water). The mixture was pulsed with an ultra-sonic probe for four, 15 second pulses, which was followed by constant agitation for 15 minutes. The mixture was then gravity filtered through a course material and vacuum

filtered to remove all insoluble materials. The extract was then rotory-evaporated at 40^0 C, frozen, and lyophilized. Dried crude extract was stored at -20^0 C.

2.4 Primary fractionation of crude extract and pre-treatment procedures

Crude extract was pre-adhered to silica gel prior to chromatography. Extract was dissolved in minimal MeOH, manually added to and mixed onto the silica gel in small batches, and lyophilized to remove solvent. Mass ratio of extract to silica was 1:2. Initial fractionation intended for bioassay was performed on a 50 mm flash column loaded with 100 g silica gel prepared in a 80/20 DCM/MeOH slurry. 18 g pre-adhered sample was loaded on a minimum solvent bed and eluted with 80/20 DCM/MeOH. 500 ml fractions were collected for a total of seven fractions. The first fraction was occasionally cut short to remove colored components. Fractions were assessed by thin layer chromatography (TLC), dried by rotary-evaporation, and stored at -20^0 C.

TLC of the initial fractions was evaluated with multiple mobile phase compositions on standard silica gel plates to accommodate the broad range of polarities and relative complexity of the extract. TLC was performed with 80/20, 85/15, and 92/8 ratios of DCM to MeOH, respectively. Fractions were assessed in series to facilitate comparison of relative retention factors and the presence of main components in multiple fractions.

Once the first fraction was identified as the primary focus for subsequent separations, the same method was applied as a pre-treatment step to reduce sample complexity. In the case of sample pre-treatment, up to 60 g pre-adhered sample was loaded. Following collection of the first fraction, the column was flushed with MeOH. Pre-treatment columns were run until 5.6 g of fraction 1 had been collected.

2.5 Secondary fractionation of pre-treated samples

The second phase of fractionation was performed on a 50 mm flash column, loaded with 100 g silica gel prepared in a 92/8 DCM/MeOH slurry. 5.6 g of pre-treated fraction was loaded in minimal solvent, pressed into the column, and eluted with 1200 ml

92/8 DCM/MeOH, collecting 60 ml fractions. The mobile phase was then changed to 500 ml MeOH, collecting 50 ml fractions. TLC was performed on fractions 1-23 using 92/8 DCM/MeOH, and on fractions 22-34 using 85/15 DCM/MeOH. Fractions were assessed for relative complexity and homology, grouped based on overlapping components, rotary-evaporated for storage and quantification, and assayed for the inhibitions of nSMase and NOX activities.

Multiple fractionations were performed in the isolation process. The fact that each separation produced between 7 to 30 fractions, and the fact that small shifts occurred in the elution of target compounds, necessitated the development of a numbering scheme to describe each unique fraction. Each fraction was assigned a value based on its relative elution in each step of the isolation. For instance, if a fraction was eluted in the first position of the first chromatographic separation, the 18^{th} position of the second separation, and the 9^{th} position of the final separation, it would be named fraction 1,18,9. Additionally, if multiple fractions were combined to consolidate a particular target, the range would be addressed. For instance, if in the final separation of the prior example the target compound was in fractions 8,9, and 10, and was combined to increase the yield, the sample would be called 1,18,8-10. All active compounds described were isolated from the first fraction.

2.6 Tertiary fractionation: Isolation of DPG and blueberric acid

Fraction 1,28 demonstrated inhibitory activities for nSMase and NOX. Further purification was achieved via flash chromatography. The sample was loaded onto a 20 mm flash column loaded with 17.6 g of silica gel. Fractions 1-12 were eluted with 85/15 DCM/MeOH in 10 ml increments. Fractions 13-19 were eluted with MeOH in 10 ml increments. Fractions were assessed on reverse phase TLC using 33/67 acetone/water. Fraction 1,28,8 was selected for testing by NMR and LC-MS, yielding DPG and blueberric acid. ^{1}H, ^{13}C, DEPT, gCOSY, gHSQC, and gHMBC NMR were performed in d_4MeOH. LC-MS was performed in positive and negative ion mode using electrospray ionization (ESI) and a quadrapole, time-of-flight detector (Q-TOF).

DPG and blueberric acid were further purified for MS and bioassays via HPLC. The presence of four carboxylic acid groups in blueberric acid make it resistant to interaction with reverse phase stationary phase in conjunction with an aqueous mobile phase. HPLC was performed on a 250 x 4.6 mm C-18 column. The mobile phase was 20/80 ACN/water and was run in an isocratic fashion at 1 ml/min. Blueberric acid eluted with the solvent front and DPG eluted at 4.1 min.

2.7 Tertiary fractionation: Isolation of malic acid

Fraction 1,18-19 demonstrated inhibitory activities for nSMase and NOX. Further purification was achieved via flash chromatography. The sample was loaded onto a 20 mm flash column loaded with 14.9 g of silica gel. Fractions 1-6 were eluted with 89/11 DCM/MeOH in 10 ml increments. Fractions 7-13 were eluted with MeOH in 10 ml increments. Fractions were assessed on normal phase TLC using 88/12 DCM/MeOH. Fraction 1,18-19,3 was selected for testing by NMR, yielding malic acid. ^{1}H, ^{13}C, DEPT, gCOSY, gHSQC, and gHMBC NMR were performed in d_4MeOH.

2.8 Tertiary fractionation: Isolation of ursolic acid

Fraction 1,8 demonstrated inhibitory activities for nSMase and NOX. Further purification was achieved via flash chromatography. The sample was loaded onto a 20 mm flash column loaded with 16.8 g of silica gel. Fractions 1-20 were eluted with 95/5 DCM/MeOH in 10 ml increments. Fractions 21-26 were eluted with MeOH in 10 ml increments. Fractions were assessed on normal phase TLC using 95/5 DCM/MeOH. Fraction 1,8,8-9 was selected for testing by NMR, yielding ursolic acid. ^{1}H, ^{13}C, DEPT, gCOSY, gHSQC, and gHMBC NMR were performed in d_6DMSO.

2.9 Isolation of β-sitosterol

Fraction 1,6 demonstrated inhibitory activities for nSMase and NOX. Further purification was not required for identification of β-sitosterol by NMR analysis. ^{1}H, ^{13}C, DEPT, gCOSY, gHSQC, and gHMBC NMR were performed in $CDCl_3$.

3.0 Results

3.1 Initial evidence of anti-inflammatory components in the Alaska bog blueberry: Whole extract inhibition of nSMase and NOX

Initial studies demonstrated that both whole aqueous and whole organic Alaskan bog blueberry extracts inhibited nSMase activity in TNF-α treated SH-SY5Y human neuroblastomas. A 2 hour pretreatment with 5 μg/mL concentrations of extract abolished nSMase response, with results statistically indistinguishable from controls. These data prompted research for active components within the extract.

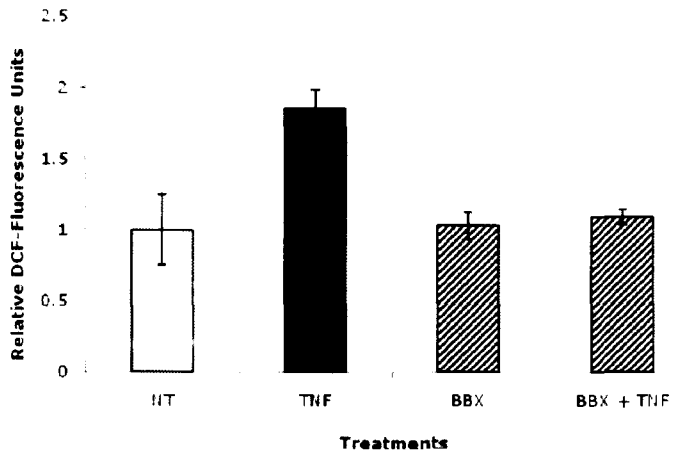


Figure 3 The inhibition of TNF-α-induced NOX inhibition via p67 translocation. Treatment of SH-SY5Y human neuroblastomas with TNF-α nearly doubled activation of NOX as determined by translocation of the p67 subunit. Treatment with whole blueberry extract had no effect on NOX activation without TNF insult, but abolished TNF-induced activation of NOX. (Kuhn Lab, University of Alaska Fairbanks.)

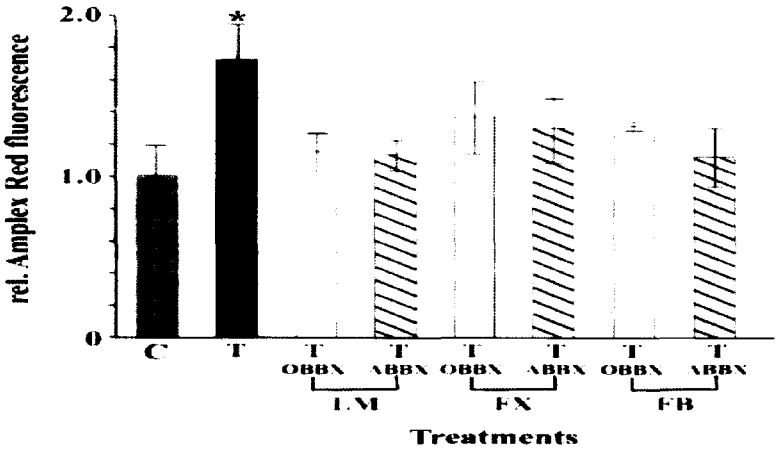


Figure 4 The inhibition of TNF-α-induced nSMase activation by organic blueberry extract (OBBX) and aqueous blueberry extract (ABBX). Whole blueberry extracts from three regions (Lake Minchumina (LM), Fox (FX), and Fairbanks(FB), Alaska) showed inhibition of nSMase activation versus the TNF-treated neurons (T), with LM berries inhibiting nSMase to levels statistically comparable to those of the control (C). (Alaska Wild Blueberry Extracts Inhibit a Magnesium-Dependent Neutral Shingomyelinase Activity in Neurons Exposed to TNFα.)

Initial screening of nSMase inhibitory activity in fractions from normal phase flash chromatography, described in the methods section, showed a significant inhibition in the first fraction (data not shown). This observation was surprising, because the early fractions excluded the prevalent anthocyanidins and other compounds which had received attention in earlier publications. It also provided the rationale for sample pretreatment and scaling up for subsequent separations.

3.2 Fractionation of Alaska bog blueberry and identification of biologically relevant secondary metabolites: Tracking via inhibition of nSMase and NOX

The primary fraction was subjected to additional fractionation. Fractions were analyzed via thin layer chromatography (TLC) and assessed for mixture complexity, and

grouped for bioassay based on redundancy to increase yield in subsequent separation steps. Each group of fractions was assayed for inhibitory activity against TNF-α stimulated nSMase and NOX activation in SH-SY5Y human neuroblastomas, with results shown in Figure 5. It is important to note that some fractions were more potent at inhibiting one enzymatic system over another, and this trend held for isolated compounds as well.

Additional chromatographic steps, previously described in the Methods section, were guided by a combination of mixture complexity, bioassay results, and the concentration of components in the active fractions. Unfortunately, not all active fractions had sufficient yield to determine structures. Ultimately, the bioassay guided fractionation produced sufficient quantities of five natural products for structural identification, including two not previously described in the literature. Two triterpenes were identified, one polyphenolic compound, and two small organic acids. Fraction 6 yielded β-sitosterol (SS), fraction 8 yielded ursolic acid (UA) and small quantities of oleanolic acid (OA), fraction 18 yielded malic acid (MA), and fraction 28 yielded both novel compounds, blueberric acid (BBA), and a dimeric protocatechuic acid glycoside (DPG).

All isolated compounds were assayed for specific activities at 5 μg/mL. Purchased standards were also assayed for comparison, when applicable. Results are shown in Figures 6 and 7. β-sitosterol demonstrated 74.4% inhibition of nSMase activation and 86.9% inhibition of NOX activation by p67 translocation. Ursolic acid demonstrated 61.5% inhibition of nSMase and 89.4% inhibition of NOX. Malic acid effectively inhibited NOX activation by 48.4% and inhibited nSMase by 67.9%. BBA abolished nSMase activation with 101.7% inhibition, and only moderately inhibited NOX by 30.6%. DPG proved to be a moderate inhibitor of both nSMase and NOX activation, with 56.5% and 25.0% inhibition, respectively.

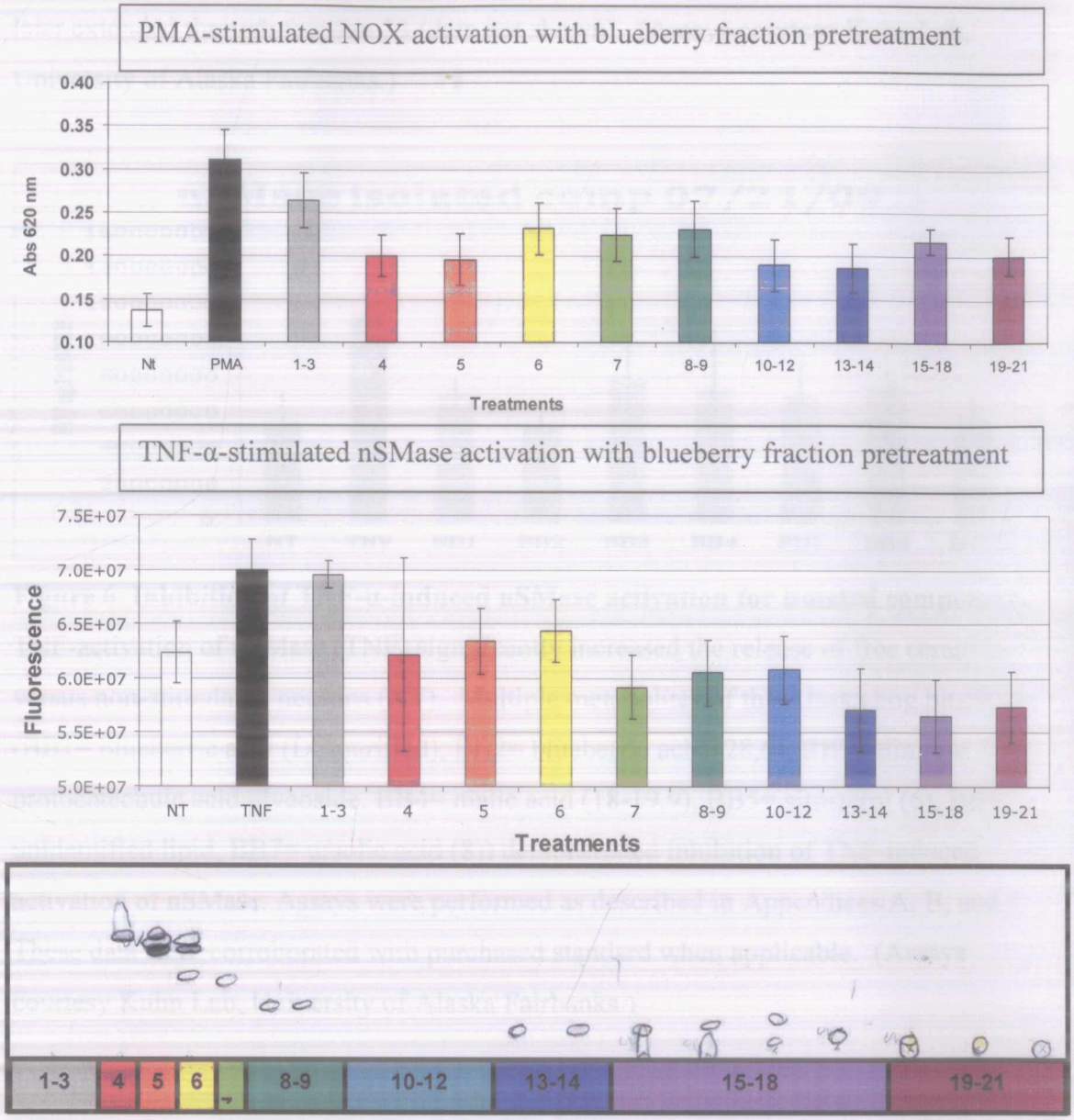


Figure 5 Comparison of TNF-α-stimulated nSMase and NOX inhibition by fractions of blueberry extracts. Fractions were grouped for bioassay via normal phase TLC analysis utilizing a 92/8 ratio of DCM to MeOH. Boths assays demonstrate the capacity to discriminate for active components versus via fractionation. Multiple fractions in both assays demonstrate inhibitory activities as compared to TNF-insulted neurons (TNF) and even suppressed levels below non-treated (NT) conditions. Assays

were performed as described in Appendices A, B, and C. Combined fractions 19-21 were later extended through fraction 33 (data not shown). (Assays courtesy Kuhn Lab, University of Alaska Fairbanks.)

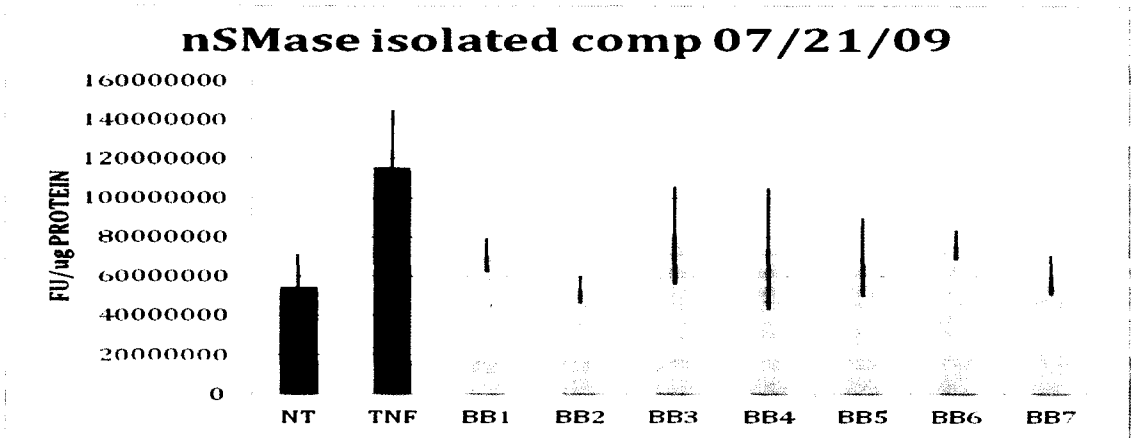


Figure 6 Inhibition of TNF-α-induced nSMase activation for isolated components. TNF-activation of nSMase (TNF) significantly increased the release of free ceramides versus non-stimulated neurons (NT). Multiple metabolites of the Alaska bog blueberry (BB1= blueberric acid (LC purified), BB2= blueberric acid (28,6), BB3= dimeric protocatechuic acid glycoside, BB4= malic acid (18-19,9), BB5= sitosterol (6), BB6= unidentified lipid, BB7= ursolic acid (8)) demonstrated inhibition of TNF-induced activation of nSMase. Assays were performed as described in Appendices A, B, and C. These data were corroborated with purchased standard when applicable. (Assays courtesy Kuhn Lab, University of Alaska Fairbanks.)

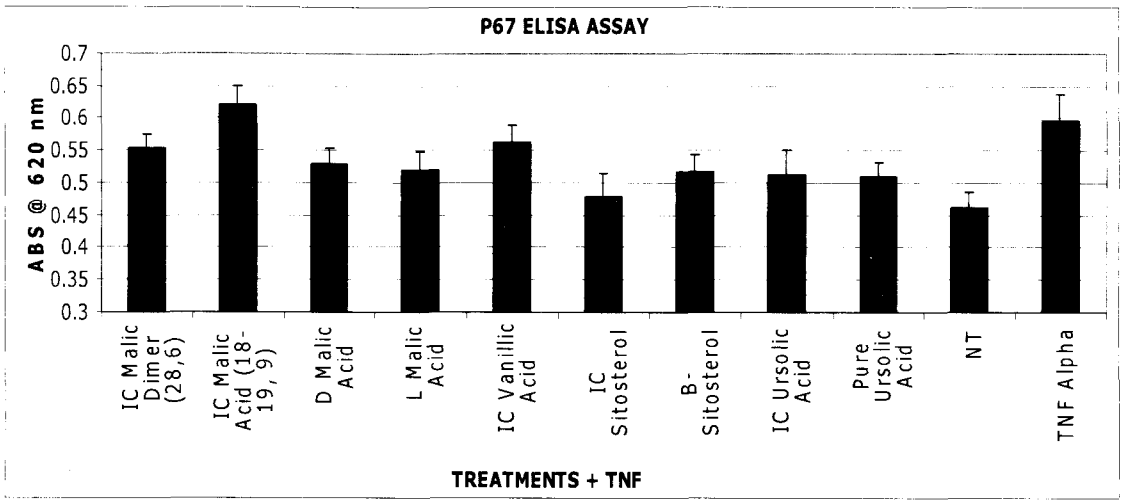
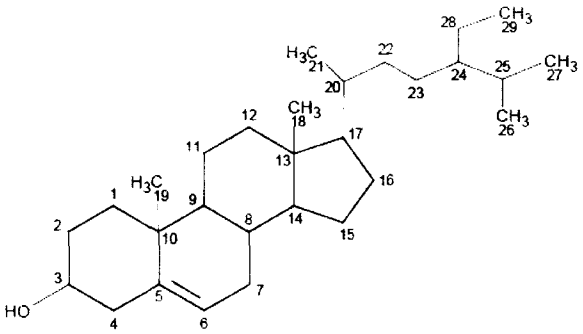


Figure 7 Inhibition of TNF-α-induced NOX activation for isolated (IC) and purchased standard components. Multiple purified components of Alaska bog blueberries demonstrate inhibition of p67 translocation in the formation of functional NOX enzyme. Blueberric acid (IC malic dimer) and the protocatechuic acid dimer (IC vanillic acid) demonstrate moderate inhibition, while pre-treatment with β-sitosterol abolishes TNF-induced translocation to levels comparable to controls (NT). Inhibition of TNF-induced NOX activation (TNF) is demonstrated for both isolated compounds and purchased standards, when applicable. (Assays courtesy Kuhn Lab, University of Alaska Fairbanks.)

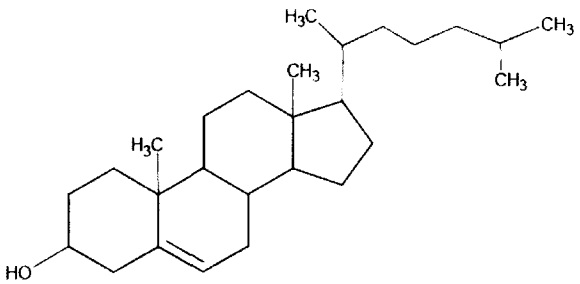
3.3 β-Sitosterol: Structure and analysis of NMR

β-sitosterol, a tetracyclic triterpene, was found in fraction 6. The structure was determined by NMR analysis of the unknown, verification by published NMR, and verification by NMR of purchased standards. NMR was performed in $CDCH_3$ on a 300 MHz Mercury instrument. Analysis of the ^{13}C and DEPT spectra predicted the degree of saturation and number of rings, number of double bonds, number of hydroxyls, and number of acids. Known molecules meeting the criteria were screened to narrow the search. Once the candidate structure was proposed, the ^{13}C spectra was compared to published data (Moghaddam et al. 2006), and was in excellent agreement as shown in Table 1. NMR obtained from authentic β-sitosterol (obtained from Sigma) was also in

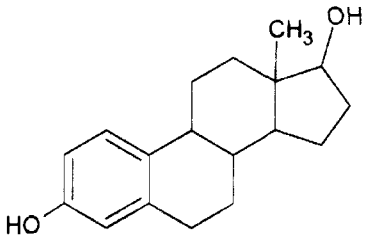
excellent agreement. The fact that β-sitosterol demonstrated inhibition of neuroinflammatory enzymes was surprising due to the ubiquitous nature of the molecule; however, recent insights into the implications of lipid raft formation and composition on neuroinflammation may shed light on these results. β-sitosterol may integrate into lipid rafts, affecting the structure, and inhibiting the formation of multiple protein complexes associated with inflammation (Gustafson et al. 2010). This is plausible due to the degree of structural similarity between β-sitosterol and dietary cholesterol, a principle component of lipid raft structure. Additionally, levels of β-sitosterol are especially high in olive oil and various nuts which have been associated with a myriad of health benefits.



β-sitosterol



Cholesterol



Estradiol

Figure 8 Structure of β-sitosterol, cholesterol and estradiol. β-sitosterol is structurally very similar to dietary cholesterol and hormonal sterols, including estradiol, and acts as a precursor in plant sterol synthesis. It is the most prevalent dietary phytosterol and has demonstrated anti-inflammatory characteristics in multiple experimental models.

Table 1 A comparison of published and observed ^{13}C NMR, gHSQC and gHMBC for β-sitosterol. Observed ^{13}C spectra are in excellent agreement with published values. Short and long-range couplings are in accordance with those predicted from the structure.

Carbon #	Published ^{13}C ppm	Observed ^{13}C ppm	C-H gHSQC ^{1}H ppm	C-H gHMBC (8 hz) ^{1}H ppm
1	37.7	37.22	1.84	1.05
2	32.3	31.88		
3	72.2	71.82	3.56	
4	42.8	42.31	2.25	5.39
5	141.2	140.72		1.05
6	122.1	121.56	5.39	
7	32.1	31.56		
8	32.3	31.88		5.39
9	50.6	50.10		1.05
10	36.9	36.49		5.39
11	21.5	21.07		
12	40.2	39.75		0.72
13	42.8	42.20		
14	57.2	56.75		0.72
15	24.7	24.30		
16	28.7	28.25		
17	56.5	56.03		0.96, 0.72
18	12.4	11.98	0.72	
19	19.8	19.40	1.05	
20	36.6	36.14		
21	19.2	18.78	0.96	
22	34.4	33.89		0.96
23	26.5	26.02		
24	46.2	45.80		0.89, 0.85
25	29.6	29.12		
26	20.2	19.84	0.87	0.85
27	19.5	19.03	0.85	0.87
28	23.5	23.05		
29	12.3	11.86	0.89	

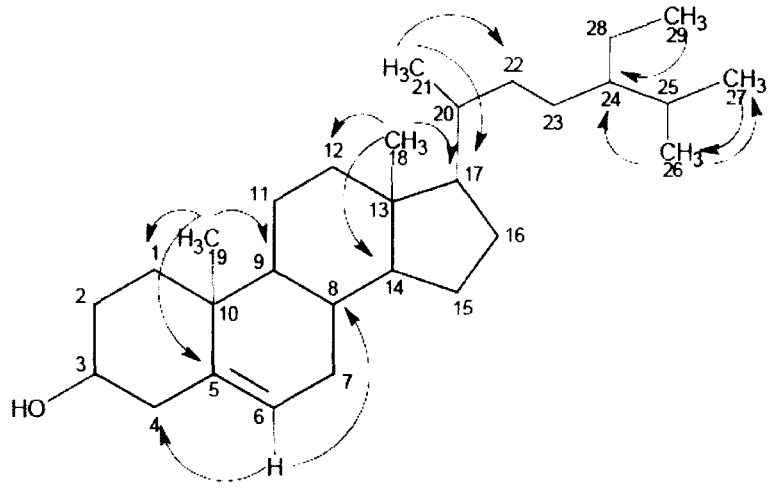


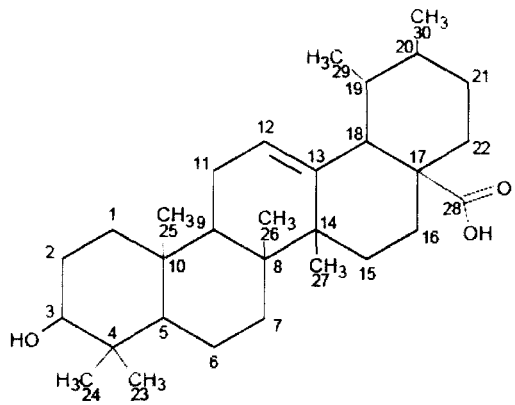
Figure 9 β-sitosterol long-range C-H couplings via gHMBC. Only select correlations are shown to methyl protons with the exception of H6. A majority of the CH_2 resonances are convoluted and therefore not addressed.

3.4 Ursolic acid: Structure and analysis of NMR

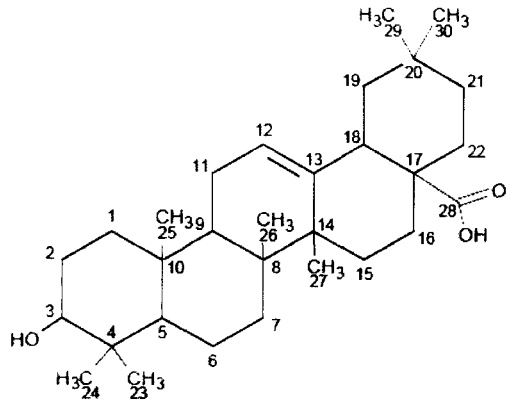
Ursolic acid, a pentacyclic triterpene. was found in fraction 8. A relatively small quantity of oleanolic acid was also found. The structure was determined by NMR analysis of the unknown, verification by published NMR (Moghaddam et al. 2006), and verification by NMR of purchased standards (Sigma). NMR was performed in deutero-DMSO on a 300 MHz Mercury instrument. Analysis of the ^{13}C and DEPT spectras predicted the degree of saturation and number of rings, number of double bonds, number of hydroxyls, and number of acids. Known compounds meeting the criteria were screened to narrow the seaerch. Once the candidate structure was proposed, the ^{13}C spectra was compared to published data, and was in excellent agreement as shown in Table 2.

Small quantities of oleanolic acid are likely present in the Alaska bog blueberry. Oleanolic acid is commonly found in conjunction with ursolic acid, and proved difficult to separate, even by LC. The NMR was very similar in ^{13}C, exhibiting significant overlap. However, critical differences in the 2D NMR allow for rapid discrimination

between ursolic and oleanolic acid, provided there are no other interfering compounds obscuring the spectra. The most notable difference between the spectra of the two structures include the gHSQC and gHMBC resonances at the H18 and C18 positions, which avoid obfuscation due to the proximity to shielding groups. Comparison to a published determination of ursolic and oleanolic mixtures suggests the presence of low levels of oleanolic acid in the Alaska bog blueberry (Kontogianni et al. 2009). Spectra are shown in the supplementary materials. Oleanolic acid was not chosen for further investigation because it was a minor component relative to ursolic acid and had significantly less research supporting it as a known bioactive molecule.



Ursolic Acid



Oleanolic Acid

Figure 10 Structure and number scheme for ursolic acid (UA) and oleanolic acid (OA). The only difference between the two terpenes is the location of the methyl group on C19. The molecules are difficult to separate by column chromatography and are regularly found together in natural product isolations.

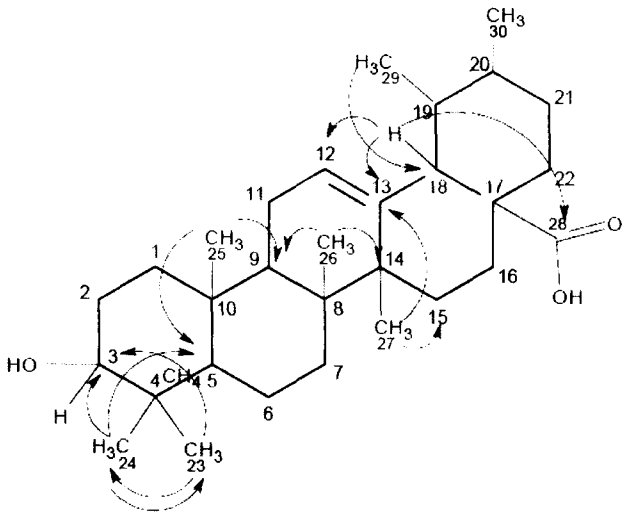


Figure 11 Ursolic acid (UA) long-range C-H couplings via gHMBC. Only select correlations are shown to methyl protons with the exception of H18. A majority of the CH2 resonances are convoluted, and therefore not addressed.

Table 2 A comparison of published and observed ^{13}C NMR, gHSQC and gHMBC for ursolic acid (UA). Observed ^{13}C spectra are in excellent agreement with published values. Short and long-range couplings are in accordance with those predicted from the structure.

Carbon #	Published ^{13}C ppm	Observed ^{13}C ppm	C-H gHSQC ^{1}H ppm	C-H gHMBC (8 hz) ^{1}H ppm
1	39.2			
2	27.6	27.44		
3	77.7	77.26	3.00	0.89, 0.68
4	39.2			
5	55.6	55.22		0.89, 0.87, 0.68
6	18.9	18.45		
7	33.6	33.14		
8	40.0			
9	47.9	47.45	1.40	0.8, 0.75
10	37.4	37.76		
11	23.7	23.30		
12	125.4	125.01	5.13	2.10
13	139.0	138.63		2.10, 1.04
14	42.5	42.08		1.04, 0.75
15	28.4	27.98		1.04
16	24.7	24.24		
17	47.7	47.26		
18	53.2	52.81	2.10	0.81
19	39.4			
20	39.3			
21	31.3	30.69		
22	37.7	36.97	1.53	
23	29.1	28.71	0.68	
24	16.1	15.68	0.89	
25	16.9	16.54	0.87	
26	17.8	17.36	0.75	
27	24.1	23.72	1.04	
28	179.1	178.75		
29	17.9	17.47	0.81	
30	21.9	21.54	0.92	

3.5 3-O-(4-hydroxyphenylcarboxylic acid) 4-O-(β-D-glucopyranosyl) gallic acid or Dimeric protocatechuic acid glycoside (DPG): Structure and analysis of NMR

NMR was performed in CH_3OD on a 300 MHz Mercury instrument. The ^{13}C and DEPT NMR spectra of DPG showed the presence of 4 subunits: 2 carboxylic acids (δ = 162.1 and 162.5 ppm), two aromatic rings having a total of 5 oxygenated carbons (δ = 134.8, 144.7, 148.4, 157.3 and 164.0 ppm), 5 protonated carbons (δ = 93.9, 98.8, 115.3, 116.5 and 122.3 ppm) and two C-substituted carbons (δ = 104.8 and 121.1 ppm), and an aldohexose tentatively assigned as a glucopyranose having a protonated anomeric carbon (δ = 103.3ppm), four O-methines (δ = 65.3 (2 carbon peak), 71.9 and 72.9 ppm) and an O-methylene (δ = 67.3 ppm).

The substitution patterns of the aromatic rings were easily deduced from the H NMR spectra. Three single hydrogen absorbances at δ = 6.96 (d; J= 7.8 Hz), 7.67 (dd; J= 7.8, 2.3 Hz) and 7.82 (d; J= 2.3 Hz) ppm were only consistent with a 1,2,4-trisubstituted aromatic ring. In addition, two single hydrogen doubles (J = 2.4 Hz) at δ = 6.26 and 6.56 ppm were consistent with a tetrasubstituted aromatic ring having two meta protons. The dimeric protocatechuic acid glycoside (Figure 12) is proposed based on chemical shift comparisons with model compounds, and HMBC correlations (Figure 13 and Table 3). The HMBC correlation between H3 and C8 defines the relative orientation of the two aromatic rings. The HMBC correlation between the anomeric hydrogen of the aldohexose (δ = 5.25 ppm; J= 6.0 Hz) and an aromatic carbon at δ = 134.8 were consistent only with it being located at C9 or C10. Since C9 is flanked by two ortho oxygens while C10 has only one ortho oxygen, the most upfield chemical shift (δ = 134.8) must correspond to C9 while C10 corresponds to the remaining downfield aromatic signal (δ = 164.0 ppm). Consequently the aldohexose is attached to C9. This conclusion was further supported by the lack of a NOESY correlation between any of the aldohexose protons and C11.

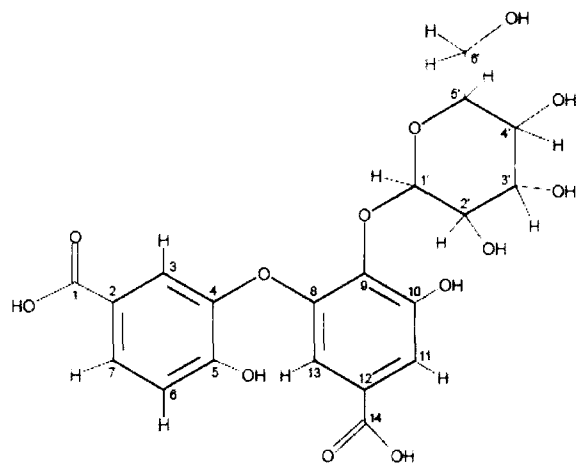


Figure 12 Dimeric protocatechuic acid glycoside (DPG). DPG is a structural dimer of protocatechuic acid caused by oxidative coupling between C4 and C8, and exhibiting glycosylation at C9.

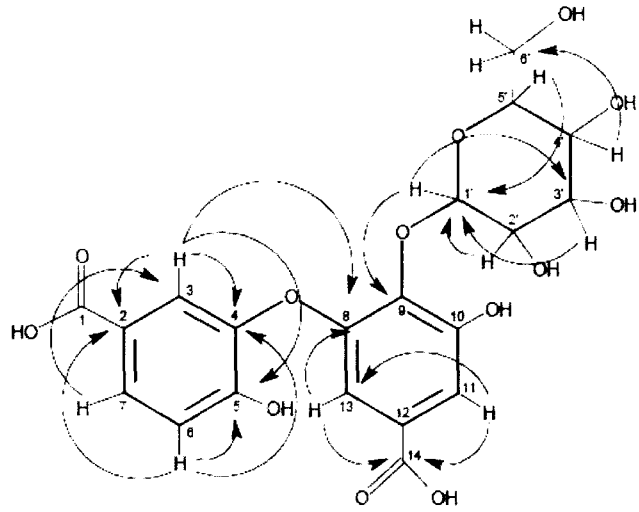


Figure 13 Selected dimeric protocatechuic acid glycoside (DPG) long-range C-H couplings via gHMBC. Both 2 and 3 bond correlation were observed to the conjugated rings. gHMBC was run at multiple frequencies to corroborate assignments. Nearly all protons and carbons were positively assigned via NMR observations.

Table 3 ^{13}C, gHSQC, and gHMBC NMR correlations of DPG. All aromatic protons were positively located via gHSQC, and relative ring orientations were determined via gHMBC.

Carbon #	^{13}C ppm	C-H gHSQC ^{1}H ppm	C-H gHMBC (8 hz) ^{1}H ppm
1	162.1		
2	121.1		7.81, 6.96
3	116.5	7.81	7.67
4	144.7		7.81, 6.96
5	148.4		7.81, 7.67, 6.96
6	115.3	6.96	
7	122.3	7.67	7.81
8	157.3		7.81, 6.56
9	134.8		5.25
10	164.0		6.56, 6.26
11	98.8	6.26	6.56
12	104.8		6.56, 6.26
13	93.9	6.56	6.26
14	162.5		6.26
1'	103.3	5.25	3.59, 3.78, 3.43
2'	71.7	3.93	3.71
3'	65.3	3.76	
4'	72.9	3.71	3.95, 3.78
5'	65.3	3.43	5.25
6'	67.3	3.83	3.71

3.6 Dimeric protocatechuic acid glycoside (DPG): Discussion of MS

Mass spectroscopy (MS) on DPG supports the proposed structure (Figure 14). LC- MS was performed on a Q-TOF-ESI at the Washington University Resource for Biomedical and Bio-organic Mass Spectroscopy. LC was performed on a C-18, reverse phase column with an isocratic, 100% MeOH, mobile phase. The TOF acquisition was calibrated, and high resolution spectrum was aquired, over a 20-1000 m/z range. All peaks present were singly charged. The theoretical molecular ion for the compound, at 468.0904 m/z, was not detected in positive ion mode, but the main peak positively matched the theoretical [M-2CO+Na-H+Na]+ m/z at 457.0722 m/z within 1.0 ppm. The secondary peak matched the same ion with a deuterium in place of a hydrogen. This is not surprising, considering the same sample was used previously in NMR analysis.

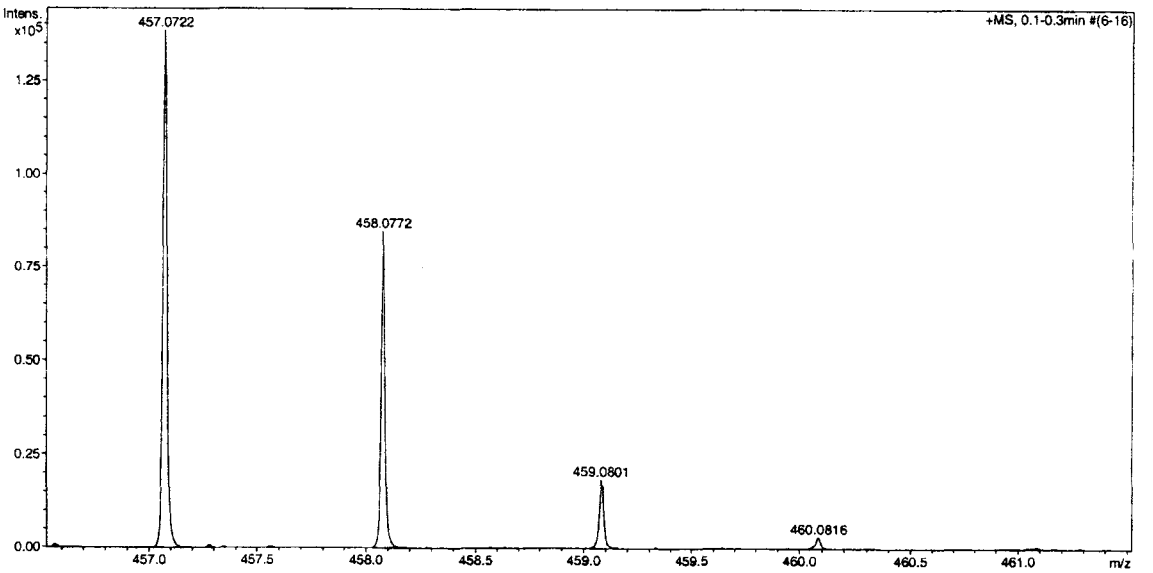


Figure 14 Positive ion mass spectrometry of DPG. Results of Q-TOF_MS with an electrospray ionization source. The main peak at 457.0722 m/z matches the theoretical [M-2CO+Na-H+Na]+ m/z, and the secondary peak matches the mass predicted from the addition of deuterium from NMR solvent.

3.7 Malic acid: Determination of structure via NMR

Malic acid was identified in fractions 18 and 19. NMR was performed in CH_3OD on a 300 MHz Mercury instrument. The NMR analysis was straight forward due to the relative simplicity of the molecule. The structure was confirmed via comparison to NMR of purchased standards (Sigma).

3.8 2,3-dihydroxybutane-1,2,3,4-tetracarboxylic acid or blueberric acid (BBA): Structure and analysis of NMR

NMR was performed in CH_3OD on a 300 MHz Mercury instrument. The proton NMR spectrum for BBA (included in Supplementary Materials) was a remarkably simple doublet of doublets, resulting from coupling between the diasteriotopic H3 protons. The hydroxyl and carboxylic acid protons were not resolved in d-acetone. Only four carbons resonances were visible, due to the symmetry present in the molecule. The long-range gHMBC coupling between the H3 and C3 also implies symmetrical structure (Figure 16).

The structure of each symmetrical half of BBA is very similar structurally to malic acid (Table 4), lacking only the proton at C2, and the ^{1}H and ^{13}C NMR also demonstrate this similarity. H3 protons are shifted slightly downfield in reference to the malic acid equivalent, as are the C2 and C3 carbons. These slight shifts comply with theoretical predictions due to increased deshielding at these positions. Most importantly the malic acid proton at 4.47 ppm is no longer present, and the coupling from the doublet of doublets at H3 to that position is also no longer present. The only possible structures include the tetracarboxylic acid and cyclic products of dehydration reactions. This question was addressed via mass spectroscopy (MS) (Figure 17).

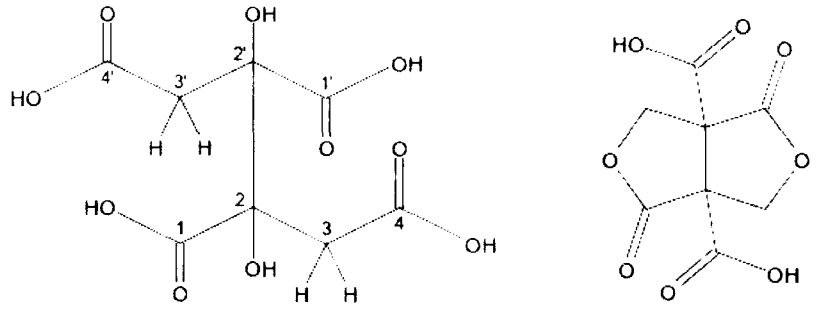


Figure 15 Blueberric acid (BBA) and a possible bicyclic product of dehydration. Two possible solutions to the structural data procured from NMR analysis. The tetracarboxylic acid structure was preferred due to observed polarity in column chromatography and was confirmed via MS. The numbering scheme used for assignment of NMR data diverges from that used in the naming for the sake of simplification due to the plane of symmetry in the molecule.

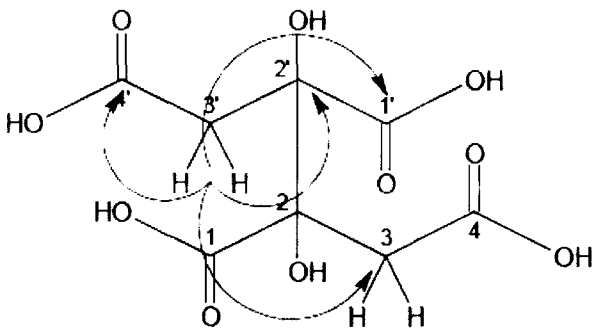


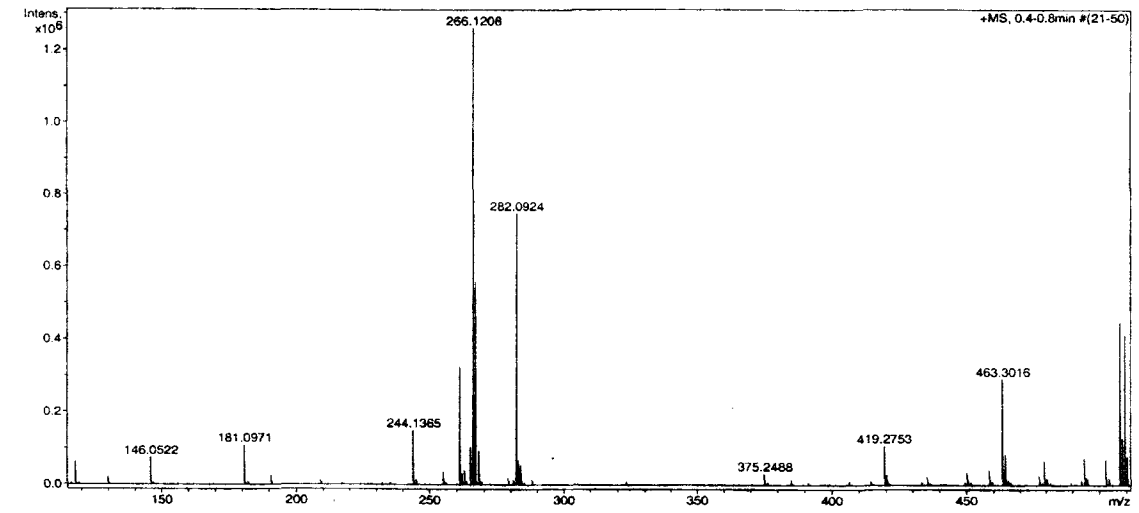
Figure 16 BBA gHMBC correlations. The notable correlation between C3 and H3 suggests a long-range coupling due to the symmetry. All four carbons correlate to H3 in gHMBC. Symmetrical correlations are not illustrated for clarity's sake.

Table 4 Observed ^{13}C resonances, gHSQC, and gHMBC correlations of BBA. The symmetrical sections of BBA show excellent homology with analogous positions of malic acid. The slight downfield shifts in C2 and C3, relative those of malic acid, are predicted due to increased shielding effects in BBA. Unlike malic acid, in BBA all four unequivalent carbons correlate to H3 in the gHMBC, due to the symmetrical structure.

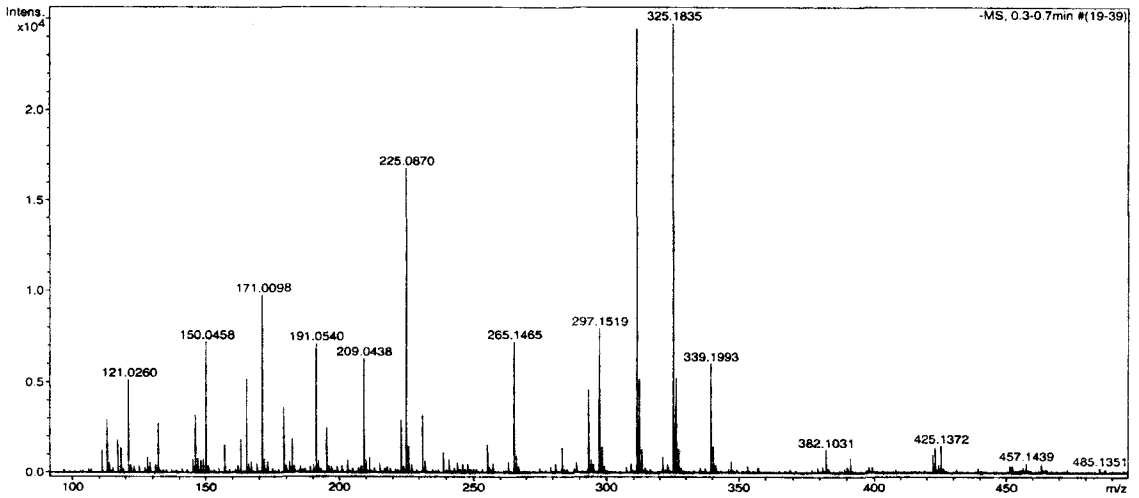
Carbon #	Malic Acid ^{13}C ppm	Observed BBA ^{13}C ppm	C-H gHSQC ^{1}H ppm	C-H gHMBC (8 hz) ^{1}H ppm
1, 1'	175.45	174.32		2.89
2, 2'	67.28	72.99		2.89
3, 3'	38.81	42.40	2.89	2.89
4, 4'	173.05	171.03		2.89

3.9 Mass spectroscopy of BBA

Mass spectroscopy of BBA was performed on a quadrapole time-of-flight (Q-TOF) MS with ESI in both positive and negative ion detection. The Washington University Resource for Biomedical and Bio-organic Mass Spectroscopy performed the analysis. LC was performed on a C-18, reverse phase column with an isocratic, 100% MeOH, mobile phase. The TOF acquisition was calibrated, and high resolution spectrum was aquired, over a 20-1000 m/z range. All peaks present were singly charged. Although, neither confirmed the molecular ion of the predicted structure within desired limits, the results confirm the non-cyclic structure presented. The observed singly charged, positive ion of 226.1208 m/z was only 0.09 m/z from the predicted molecule [M+] (Figure 17). Although only a minor peak in the negative ion mode, the peak at 265.1465 m/z represents [M-H].



Positive Ion Detection MS



Negative Ion Detection MS

Figure 17 Positive and negative ion Q-TOF mass spectrum of BBA. The observed singly charged, positive ion of 226.1208 m/z is only 0.09 m/z from the predicted molecule [M+]. Although a minor peak in the negative ion mode, the peak at 265.1465 m/z represents [M-H]. The molecule may be a poor candidate for negative mode detection due to extensive fragmentation.

The slight discrepancy may be due to a number of instrumental factors, particularly calibration. MS is inherently less linear over a broad range than many other quantitative techniques, and BBA would be on the small side for a molecule analyzed on

this system. This may explain the slight disagreement between predicted and measured values, especially if a broad calibration was used.

4.0 Discussion

4.1 Ursolic Acid

Terpenes are ubiquitous natural products which are biosynthetically produced from repeating units of isoprene (Figure 18). The triterpenes are 30 carbon molecules which are produced from the enzyme-catalyzed cyclization of squalene, resulting in poly-cyclic molecules, which include phytosterols as well as other biologically relevant molecules.

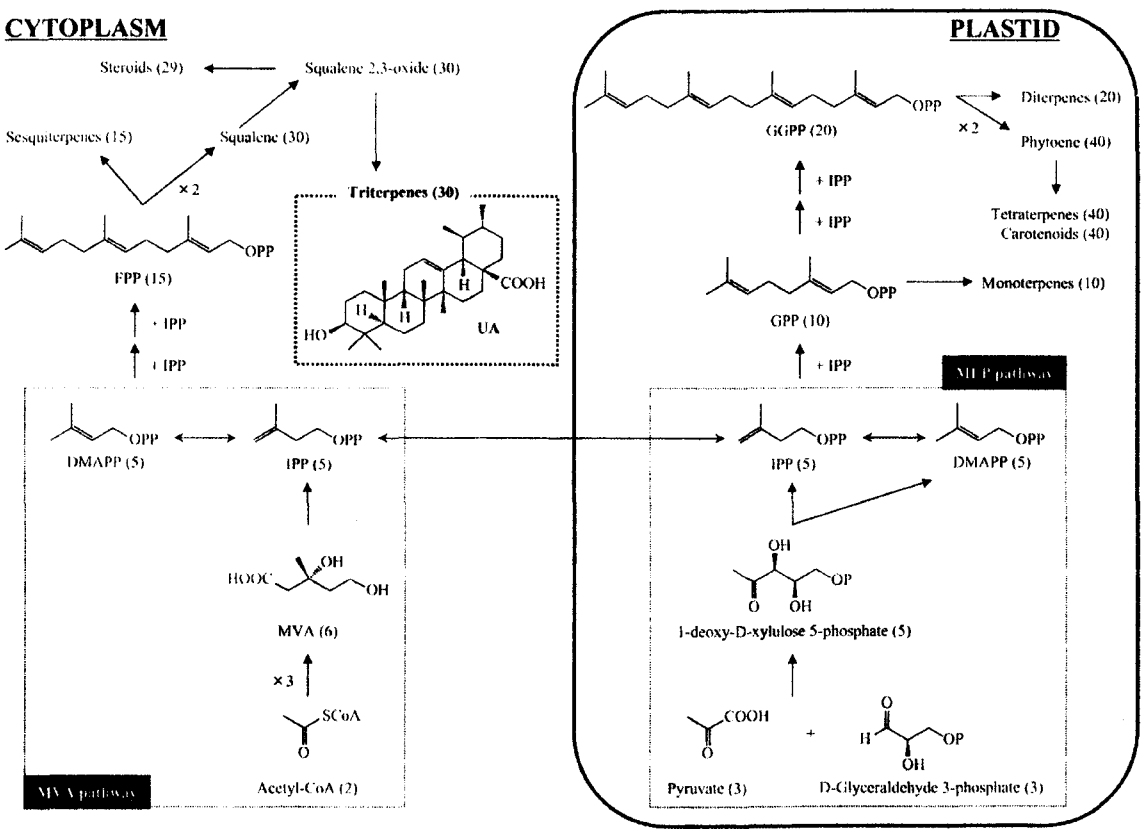


Figure 18 Terpenoid biosynthesis. Synthesis within plants between the cytosol (MVA pathway), and plastid (MEP pathway), and ursolic acid biosynthesis. Isoprene units from multiple sources are coupled forming squalene. Squalene is cyclized and modified forming polycyclic triterpenes including ursolic acid. IPP = isopentyl diphosphate, DMAPP = dimethylallyl diphosphate, FPP = farnesyl diphosphate. (Ikeda, et al., Mol. Nutr. Food Res. 2008a)

Ursolic acid, a pentacyclic triterpene used medicinally in Asia, has been shown to possess anti-oxidative, anti-inflammatory, hemoprotective, and anti-carcinogenic activities (Ikeda et al. 2008a) Folk remedies incorporating ursolic acid include Labrador tea, a medicinal beverage indigenous in Northern latitudes. The leaves of plantain, thyme and coffee have also been used as wound dressings for perceived anti-inflammatory properties, and contain high concentrations of ursolic acid. The frequent, low-dose, dietary consumption of ursolic acid is an alternative explanation as to health benefits associated with the Mediterranean diet and the French paradox, which are classically attributed to consumption of polyphenolic compounds including anthocyanins and stilbenoids.

Like dietary polyphenols, ursolic acid appears to be a metabolic double-edged sword. There is a significant body of evidence for it possessing both protective and apoptotic effects. The specific cellular response seems to depend on the mode of delivery, the concentration, treatment duration, and cellular type and stress. The fine line at which ursolic acid switches roles is complex and multi-factorial, and requires a case by case assessment.

Ursolic acid can both attenuate and induce both iNOS and COX-2 through NF-kβ suppression in LPS treated macrophages, but other studies have shown that treatment of macrophages with ursolic acid induced iNOS and TNF-α production via NF-kβ activation (Ikeda et al. 2006). Along these same lines, ursolic acid treatment has been shown to increase levels of NF-kβ-inducible IL-1β, IL-6, and MIF in peritoneal macrophages. It appears that the effect of ursolic acid has on cells depends on the dose, biological status of the cell, and whether the ursolic acid is in aggregate form.

Macrophage migration inhibitory factor (MIF) is a cytokine which regulates the activation of macrophages and induces TNF-α and COX-2. Possibly of even greater importance, MIF has been shown to inactivate p53, making it a key player not only in inflammatory processes but also tumorigenesis. RAW264.7 macrophages treated with ursolic acid in DMSO demonstrate increase release of MIF and decreased intracellular

levels of MIF without altering the expression (Ikeda et al. 2005). The increase in MIF release was shown to be linked to activation of MEK1/2 and ERK1/2.

Treatment of peritoneal macrophages with aggregated ursolic acid demonstrated induction of IL-1β. Aggregated ursolic acid is recognized by the CD36 receptor on the cell surface of the macrophages which leads to the activation of NOX and increased production of ROS (Ikeda et al. 2007). This triggers MAPK cascades, as demonstrated by increased phosphorylation of ERK1/2, p38 and caspase-1, and induction of proIL-1 β. Caspase-1 converts proIL-1 β to active IL-1 β which is then released. Macrophage susceptibility to ursolic acid induced IL-1 β release was shown to vary between strains and primarily depended on intracellular generation of O_2^- via NOX (Ikeda et al. 2008b). Essentially, the cellular redox response to the ursolic acid insult determines the extent of inflammatory cytokine production.

Apoptosis, programmed cell death, is an essential component for the healthy function of multicellular organisms; however, abnormalities in apoptotic function can lead to a variety of pathologies, including cancer. Apoptosis includes nuclear DNA degradation followed by nucleosomal fragmentation. Caspases induce some of the physical transformations which take place during apoptosis and are considered central executioners of the process. An additional component is the p53 gene, which disfunction is linked to most tumors. The p53 hold the cell in check following DNA damage, and if the damage is irreparable, apoptosis ensues. Bcl-2, a pro-survival gene, has been shown to inhibit cell death and is mediated, in part, by NF-kβ. It is important to note to NF-kβ simultaneously regulates inflammatory cytokines including TNF-α, IL-1 β, and IL-6. Ursolic acid has been shown to inhibit NF-kβ function via inhibition of both IKβ α kinase and p65 phosphorylation (Shishodia et al. 2003).

Treatment of B16F-10 melanoma cells with nontoxic concentrations of ursolic acid produced the formation of apoptotic bodies and increased levels of DNA fragmentation (Manu and Kuttan 2008). Transcription of the pro-apoptotic genes, p53 and caspase-3, were up-regulated while the levels of the anti-apoptotic gene bcl-2 was suppressed. NF-kβ was significantly inhibited as well as the downstream transcription of

inflammatory cytokines TNF-α, IL-1β, and IL-6. Taken together, ursolic acid initiates apoptosis by suppressing NF-kβ mediated bcl-2 expression and subsequently activating the TNF-α mediated pro-apoptotic genes p53 and caspase-3.

The strong anti-inflammatory properties attributed to ursolic acid may be due to inhibition of secretory phospholipase A2 (sPLA2) enzymes which participate in arachidonic acid release in inflammation and digestive physiologies. Ursolic acid inhibited a number of sPLAs isolated from both snake venoms and human synovial and pleural fluids in a concentration dependent manner (Nataraju et al. 2007). Group IIA sPLAs were inhibited 96% at 18uM concentration of ursolic acid. Fluorescent spectroscopy showed increased fluorescence of sPLAs in the presence of ursolic acid, and UV circular dichroism spectroscopy also showed a shift in absorbance for sPLAs in the presence of ursolic acid indicating a direct interaction between the two. This suggests that ursolic acid may mediate inflammation via direct inhibition of sPLAs, reducing the hydrolysis of fatty acid signaling molecules, and preventing the production of eicosanoids.

Cytochrome P450 (CYP) enzymes are involved in the metabolism of fatty acids, cholesterols, and steroids in the liver. Inhibition of CYPs is implicated in hepaprotection in scenarios of chemical and drug induced hepatoxicity. Ursolic acid and oleanolic acid, a structurally similar pentacyclic triterpene, demonstrated competitive inhibition of specific CYP isoforms in human liver microsomes (Kim et al. 2004) Ursolic acid inhibited CYP2C19 catalyzed S-mephenytoin 4'-hydroxylation, and oleanolic acid inhibited CYP1A2 catalyzed phenacetin O-deethylation and CYP3A4 catalyzed midazolam 1-hydroxylation. IC50 values for the inhibitions were 119.7, 143.5, and 78.9, respectively. The inhibition of CYPs by ursolic and oleanolic acids has implication on the rate of drug metabolism, decrease acetaminophen and ethanol hepatoxicity, and possible anti-carcinogenic effects.

PC12 cells exposed to either H_2O_2 or 1-methyl-4-phenylpyridinium ion (MPP+) demonstrated decreased viability, significant decline in GSH, and increased levels of malonyldialdehyde (MDA). Anti-oxidative enzymes glutathione peroxidase (GPX),

catalase, and superoxide dismutase (SOD) all showed decreased activities. Additionally, levels of inflammatory cytokines IL-6 and TNF-α were significantly increased in response to the influx of redox stress (Tsai and Yin 2008). Pretreatment of the PC12 cells with ursolic and oleanolic acid prior to insult both resulted in recovered viability. Additionally, levels of cellular GSH and anti-oxidant enzyme activities increased. The accompanying inflammatory cytokine response was decreased.

It is compelling that ursolic acid demonstrates pronounced antioxidant activity despite the fact that the molecular structure doesn't support the direct scavenging of free radicals. In addition to modulating the redox status of the cell, ursolic acid has also been shown to ameliorate cognitive deficits in D-galactose treated mice (Lu et al. 2007). Mice treated with D-galactose showed behavioral impairment and deficits in learning and memory, and decreased activities of the antioxidant enzymes SOD, glutathione peroxidase (GPX), and glutathione reductase (GR), compared to non-treated mice. D-galactose treated mice also treated with ursolic acid showed improved behavioral and cognitive performance versus those not given ursolic acid. Ursolic treated mice also recovered D-galactose impaired antioxidant enzyme activities. Improvement in this neuroprotective effect is linked not only to redox enzyme activity but also to increased expression of growth associated protein GAP43 in the hippocampus of senescent model mice.

Ursolic acid has been proposed to be responsible for NOX deregulation in lipophilic fractions of Alaska bog blueberry. Treatment of TNF-α –induced SH-SY5Y human neuroblastomas with ursolic acid prevented p67 translocation and integration into functional NOX. It was shown that ursolic acid inhibited the assembly of lipid rafts essential to NOX function, as determined by analysis of ganglioside-1 distribution. These data support the notion that, despite its lack of radical scavenging capacity, ursolic acid acts as a potent anti-inflammatory compound via inhibition of NOX function (Gustafson et al. 2010).

Both ursolic acid isolated from Alaska bog blueberries and ursolic acid standards have demonstrated potent inhibition of TNF-α-stimulated ceramide release in SH-SY5Y

neuroblastomas via nSMase regulation (Gustafson et al. 2010). This inhibition is not directly at the nSMase enzyme. There is an excellent chance, considering the evidence for ursolic acid modifying lipid raft formation in NOX inhibition, that a similar mechanism is responsible for the indirect inhibition of nSMase.

We have, for the first time, identified ursolic acid in the Alaska bog blueberry and have shown that ursolic acid inhibits nSMase-mediated release of ceramides and prevents the assembly of functional NOX via the modification of lipid raft formation. We have also shown an inhibition of nSMase-mediate ceramide release. Both of these systems play crucial roles in triggering inflammatory cascades, and their inhibition in neuronal models have implications in neurodegenerative disorders. Ursolic acid has not been addressed in previous studies pertaining to the nutraceutical applications of blueberries, although it exhibits powerful anti-inflammatory and anti-oxidative mechanisms as well as neuroprotective applications. The presence of ursolic acid in the Alaska bog blueberry presents avenues for alternative explanations as to the observed health benefits of blueberry supplementation.

4.2 β-sitosterol

β-sitosterol is the most prevalent phytosterol and exhibits a structure very similar to dietary cholesterol. In human consuming a diet rich in fruits and vegetable, β-sitosterol is present in plasma in μM concentration, and has been shown to compete for absorption with dietary cholesterol. β-sitosterol has demonstrated possible therapeutic applications involving carcinogenesis, cardiovascular disorders, and immune response. It also shows potential as a potent anti-inflammatory molecule. Like ursolic acid and polyphenolic anti-oxidants, it appears to have protective effects in low doses and apoptotic effects at higher doses, and the cellular environment likely dictates the critical points at which a dose becomes toxic.

β-sitosterol, present in high concentrations in olive oil, has been shown to reduce arachidonic acid release in marcophages via the cyclo-oxygenase-2 pathway. Non-toxic doses of oxidized low density lipoprotein (oxLDL) initiates increased production of

peroxide via NOX, release of arachidonic acid, cyclo-oxygenase-2 overexpression, and prostaglandin E2 (PGE2) release in RAW 264.7 macrophages. β-sitosterol treatment reduced these pro-inflammatory effects, likely via induction of glutathione peroxidase and superoxide dismutase (Vivancos and Moreno 2008).

β-sitosterol isolated by methanol extraction of the Indian medicinal plant, Pluchea indica, demonstrated powerful anti-inflammatory properties in venom-exposed mice. Viper and cobra venom was injected intravenously at varied doses to determine the minimum lethal dose (LD50), the minimum defibrinogenating dose (MDD), a measure of blood coagulation, the minimum edema dose (MED). Phospholipase A2 (PLA2) activity was also determined for treated mice. Coinjection of β-sitosterol with venom showed significant protection against the negative actions of both viper and cobra venoms (Gomes et al. 2007). Injection of 10 μg β-sitosterol with venom increased the LD50 by 6.2 fold, the MDD 2 fold and MED 3 fold. Additionally, PLA2 activity increased 6 fold in invenomated mice. β-sitosterol treatments also demonstrated reduced levels of lipid peroxidation in invenomated mice and improved superoxide dismutase activity. β-sitosterol appears to possess powerful anti-inflammatory properties which act via the arachidonic acid pathway.

Both β-sitosterol and β-sitosterol glucoside have shown potential as therapeutic agents in the treatment of asthma, a disorder characterized by lung inflammation (Yuk et al. 2007). Ovalbumin induced asthmatic mice treated intraperitonerally with 1 mg/Kg β-sitosterol showed significant reduction in eosinophil infiltration into the bronchoalveolar lavage. Total ROS was also significantly reduced in treated mice versus controls. The inflammation associated with eosiniphil infiltration also decreased as did the expression of inflammatory proteins IL-4 and IL-5. The intraperitoneal injection was relevant, because individuals who consume a diet rich in fruits and vegetable tend to have measurable β-sitosterol in their plasma.

Supplementation of multiple sclerosis (MS) patients with 4 μM β-sitosterol caused a 24% and 27% reduction in the levels of inflammatory cytokines, TNF-α and IL-12, in peripheral blood mononuclear cells, respectively (Desai et al. 2009). In healthy

subjects, 16 μM β-sitosterol treatments also showed a 30% increase in the anti-inflammatory cytokine, IL-10, but these results were not reproduced in MS patients. These data demonstrate the ability of β-sitosterol to modulate levels of both pro and anti-inflammatory cytokines in peripheral blood mononuclear cells, and likely in other tissues as well. It also shows that the cellular environment influences the cellular response to the β-sitosterol, and that the response is multi-variant.

Epidemiological studies have demonstrated that diets rich in fruits and vegetable result in lower frequency of cancers, and this is likely due to multiple groups of phytochemicals. β-sitosterol is the most commonly consumed phytosterol, and although it is structurally similar to cholesterol, it is not synthesized endogenously in humans. In addition to anti-inflammatory properties, β-sitosterol has been shown to inhibit carcinogen-induced cancer formation in rats. Other studies have shown anti-proliferative properties in cancerous cell lines associated with the activation of the sphingomyelin cycle. Apoptosis and anti-proliferation was observed in β-sitosterol treated human leukemic U937 cells, in a concentration dependent fashion (Park et al. 2007). The increase in levels of apoptosis correlated with a down regulation of Bcl-2, increasing the Bax/Bcl-2 ratios, and the activation of caspase-3. No changes were seen in levels of Bcl-xL or Bax. Inhibition of PI3K/Akt abolished β-sitosterol induced apoptosis in U937 cell, but in MCA-102 fibrosarcoma cell, an active down regulation of the PI3K signaling pathway was shown to be essential to the induction of apoptosis (Moon et al. 2008). Phosphorylation of ERK was also shown in both cell lines, and the inhibition of ERK in the MCA-102 cells blocked β-sitosterol induced apoptosis (Moon et al. 2007).

We have demonstrated that β-sitosterol inhibits nSMase-mediated ceramide release and p67 translocation into functional NOX in SH-SY5Y human neuroblastomas. In combination with other studies showing decreased arachidonic acid pathway response, these results show that β-sitosterol may have applications as a potent anti-inflammatory molecule in certain circumstances. It is possible that the significant homology between β-sitosterol and cholesterol may have significant implications in lipid raft formation and structure as well as other systems involved in cholesterol metabolism. Although it is not

surprising that Alaska bog blueberries contain β-sitosterol, its presence offers alternative explanations as to the anti-inflammatory actions of whole blueberry supplementation.

4.3 Polyphenols: DPG in context

Polyphenols are a diverse class of naturally occurring metabolites, often originating from the shikimate pathway, which are characterized by multiple phenolic groups and extended conjugation. Chemically, polyphenols are excellent scavengers of free radical oxidants, and under certain circumstances, can also act as pro-oxidants. They also exhibit strong interactions with metal cations such as Fe^{2+}. Naturally occurring polyphenols are generally classified into three classes of derivatives: flavanoids, galloyl or hydroxybenzoic acid derivatives, and hydroxycinnamic acid derivatives. All three categories can form polymers in the form of proanthocyanidins, ellagitannins, and lignin, respectively (Figure 18). The flavanoids are the most diverse group, and are comprised of the catechins, anthocyanidins, flavonols, flavones, and isoflavones. Additionally, flavanoids are readily glycosilated. Significant homology exists between classes of polyphenols in terms of general transcriptional and translational influences, although variation between dose and cell-specific response is prevalent.

Blueberries are known to be rich in catechins, anthocyanidins, flavonols, hydroxycinnamic acids, and possess modest amounts of benzoic acid derivatives. Wild *Vaccinium uliginosum* has shown 3.5 times the flavonol (quercetin and myricetin) concentration of commercial varietals, as well as over 3 times the anthocyanidin content (Hakkinen 2000). The anthocyanidins are primarily in the form of glucosides, while the flavonols are primarily bound to galactose moieties. Regional studies have also shown that anthocyanidin concentration increases with Northern latitude, so it is likely that the Alaskan bog blueberry collected in the interior region has even high levels than previously published.

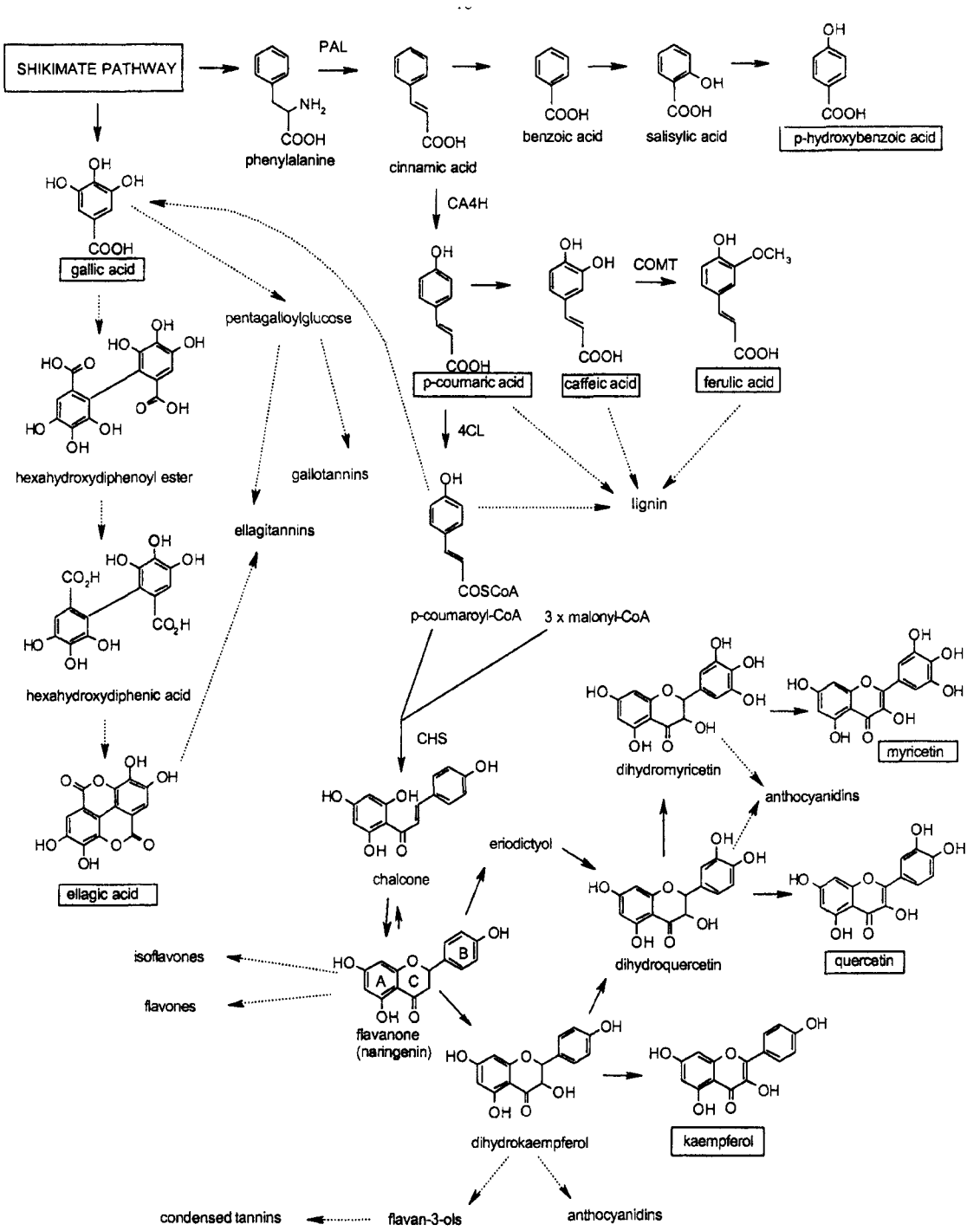


Figure 19 Biosynthesis of hydroxycinnamic acid, hydroxybenzoic acids, and flavanoids. All major classes of dietary polyphenols originate in the shikimic acid pathway and are prone to modification by polymerization and glycosylation. (S. Hakkinen, "Flavanols and Phenolic Acids in Berries and Berry Products", 2000.)

Epidemiological studies have shown individuals who consume 3-4 glasses of wine daily over a 3 year period are at 80% lower risk of dementia and Alzheimer's disease. Two cups of green tea per day were shown to significantly reduce the incidence of Parkinson's disease. Other studies on subjects over the age of 65 indicate an inverse association between gross consumption of flavonoids and dementia. In addition to showing preventative properties, Alzheimer's patients administered a standardized Ginkgo biloba extract (EGb 761) demonstrated improved cognitive function.

Consumption of green tea has shown potential therapeutic value for cancer prevention and treatment, cardiovascular disease, and inflammatory diseases. Green tea is a concentrated source of flavonoid (30% dry weight), primarily in the form of catechin derivatives, with epigallocatechin-3-gallate (EGCG) making up 60% of the total catechins present. Pretreatment of mice with either whole green tea or EGCG reduced dopaminergic response when induced by N-methy-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). It is possible that the catechol like structure of the catechin competitively inhibit the vesicular uptake of the MPTP metabolite MPP+.

In blueberries (vaccinum ashei Reade) three main classes of flavonoid are found. Catechins (387 mg / 100 g fresh weight), epicatechins (34-129 mg / 100 g fresh weight), and anthocyanins (84-113 mg / 100 g fresh weight) are all prevalent in blueberries. This makes blueberries one of the most concentrated sources of anthocyanins in commonly consumed foods. It is likely that levels are much higher in the Alaska bog blueberry based on physical observation and distribution and concentration of vivid colored components.

Most of the interesting biological effects of polyphenols are not directly attributable to their capacity to scavange free radicals. The anti-inflammatory, anti-oxidative, anti-atherogenic, and neuroprotective effects are related to a variety of transcriptional, translational, and cellular signaling effects which primarily relate to modulation of the NF-κβ, MAPK, and IGF-1 signaling pathways (Khan et al. 2006).

Catechin rich extracts showed an inhibition of Aβ induced NF-κβ activation in PC12 cells. Cytoplasmic NF-κβ consists of three subunits: the p50, p65, and the

inhibitory subunit, Iκβ. Activation of NF-κβ requires the phosphorylation of Iκβ and subsequent dissociation of the p50/p65 subunit which then translocates to the nucleus. Cytoplasmic levels of phosphorylated Iκβ were elevated in the presence of Aβ, indicating increased activation of NF-κβ. The polyphenol treatment effectively lowered the levels of pIκβ, demonstrating a decrease in active NF- κβ even in the presence of Aβ (Ramassamy 2006).

Resveratrol, a trihydroxystilbene present in red grapes, wine, and blueberries, has been shown to inhibit inflammatory cytokine production in response to liposaccharide (LPS) insult *in vivo*. In lung epithelial cell culture, resveratrol inhibited NF-κβ and activator protein 1 (AP-1) activation. Resveratrol failed to inhibit direct association of NF-κβ to DNA, but did block the TNF-α induced translocation of the p65 NF-κβ subunit. Resveratrol also inhibits the activation of c-Jun N-terminal kinase (JNK) as well as its downstream MEKs, offering an explanation as to the inhibition of AP-1.

NF-κβ is also a known regulator of the cyclo-oxygenase 2 (COX-2) and inducible nitric oxide synthase (iNOS) genes. Resveratrol has been shown to inhibit both PMA induced COX-2 and iNOS expression *in vitro*. Upstream regulation of NF-κβ is likely a primary factor in these observations.

Resveratrol also modulates MAPK signaling based on dose and cellular environment. Low levels of resveratrol induces phosphorylation of ERK 1/2 , JNK, and p38 in epidermal cells. High, but non-toxic, doses inhibit phosphorylation of MAPKs. Additionally, both the cell type and pathology have significant influence on the dose at which resveratrol becomes inhibitory to MAPK phosphorylation.

There is evidence that catechins, especially EGCG, assert their protective effects via activation of apoptotic genes. SH-SY5Y human neuroblastomas exposed to low concentrations of EGCG showed increase expression of pro-apoptotic genes such as Bax, Bad, the cell cycle inhibitor GADD45, Fas ligand, and tumor necrosis factor related apoptosis ligand (TRAIL). Anti-apoptotic genes such as Bcl-2 were not affected; however, TNF-α induced activation of PI3K and its downstream effector, Akt, has been

shown to be directly inhibited by red wine extracts and quercetin (Hwang et al. 2009). Regulation of PI3K is likely involved in the expression of apoptotic genes.

It is likely that polyphenols positively affect the cellular redox state by increasing its endogenous defenses (Ramassamy 2006). Nrf2 is a common antioxidant defense transcription factor which binds nuclear factor erythroid-derived 2 (NF-E2) binding sites which regulate erythroid specific genes. The NF-E2 falls within the group known as the anti-oxidant response elements (ARE) which are found on the promoters of multiple phase 2 detoxification genes. These elements are known to be inducible by anti-oxidants. The polyphenol alters interactions between Nrf2 and Keap1, allowing for nuclear translocation and the subsequent initiation of transcription of target genes such as heme oxygenase 1 (HO-1), AP-1, and the MAPKs.

It has been shown that oxidative stress, especially due to cigarette smoke exposure significantly reduces the effectiveness of corticosteroids. Histone actetylation and deacetylation events are key regulators of the cytokine inflammatory response. Oxidative stress affects the chromatin structure of proinflammatory genes via NF-κβ which recruits coactivators with intrinsic histone acetylation activity. Acetylation of the histone promotes unwinding of the DNA allowing transcription of the pro-inflammatory genes. Corticosteroids recruit histone deacetylase-2 (HDAC2) via the glucocorticoid receptor (GC) to the transcriptome, deacetylating the site and downregulating transcription. Oxidative stress, especially in the form of cigarette exposure, reduces the ability of GC to recruit HDAC2 while simultaneously promoting increased levels of NF-κβ (Ikeda et al. 2008a).

Dietary polyphenols have been shown to influence both NF-κβ activation and chromatin remodeling due to regulation of HDAC, thereby influencing the subsequent inflammatory response (Rahman et al. 2006). This also implies an increased sensitivity to corticosteroids which is critically important to populations with poor sensitivity.

The neurodegeneration associated with neurodegenerative diseases such as PD, AD, and ALS are multifactorial in nature. A complex set of interacting stressors cumulatively lead to programmed cell death. Oxidative damage to biomolecules,

increased inflammatory processes involving NOX and ceramides, accumulation of iron and Ca^{2+} mismanagement are all hallmarks of neurodegenerative disease.

Anti-oxidant rich extracts from blueberries, green tea and Gingko biloba have all demonstrated neuroprotective effects in *in vitro* and *in vivo* models. These actions are typically driven by regulation of MAPKs, PKC, anti-oxidant enzymes responsible for GSH metabolism, and the upregulation of survival genes. However, there is a dichotomy in the actions of polyphenols in *in vitro* models. Some studies demonstrate anti-tumorigenic, lethal properties, while others show protective, pro-survival properties. Polyphenols, especially catechins have been shown to have a biphasic mechanism based on dose. At low concentrations they tend to exert anti-oxidant, protective effects, while at high concentrations they demonstrate pro-oxidant, pro-aptotic effects. Because most anti-tumorigenic studies utilized high concentrations of polyphenols, it is unlikely that anti-tumorigenic properties can be attributed to radical scavenging qualities. Additionally, the low levels of polyphenols utilized in studies which demonstrated protective effects suggest that the results cannot be attributed to the radical scavenging qualities due to the low levels present, and more likely result from altering signaling pathways.

cDNA array based studies used to investigate the effect of EGCG dose on the cellular expression profile demonstrated the biphasic nature of the polyphenol's action (Mandel et al. 2004). Low doses (1 uM) decreased the expression of the pro-aptotic genes bax, bad, gadd45, tumor necrosis factor receptor family member fas ligand, caspases 1 and 6, and TRAIL. No changes were seen in the levels of the anti-aptotic genes bcl-2, bcl-w, or bcl-xL at low concentrations. This shows that the EGCG exerts its protective effects at low doses via down regulation of pro-aptotic genes versus the up regulation of anti-aptotic genes. At higher concentrations (50 uM) EGCG exerted clear pro-aptotic effects. mRNA levels of the pro-aptotic genes bax, bad, caspases 1 and 6, fas and fas ligand all increased.

An inverse association between polyphenol consumption and cardiovascular disease has been demonstrated, and platelet recruitment is a critical step in the activation

of platelets at the site of cardiovascular injury. Platelet recruitment is critically triggered based on the balance of platelet NO production and oxidative stress. The synergistic action of quercetin and catechin inhibited platelet recruitment in a concentration dependent manner, likely by modifying the platelet overall redox status (Pigntelli et al. 2006). Platelet recruitment and activation are directly correlated to oxidative stress as indicated by superoxide anion, and are inversely correlated to NO levels. Incubation of platlets with both quercetin and catechin significantly inhibited platelet recruitment by simultaneously increasing NO levels and decreasing O_2^-. The decrease in O_2^- was attributed to a reduction in NADPH oxidase activity via reduced platelet PKC activation. PKC has been shown to activate NADPH oxidase via the p47 subunit and is a pivotal mediator in the arachidonic acid activation of NOX. The increase in NO levels could not be attributed to NO synthase activity, and may be due to enhanced bioavailability of NO due to reduced peroxynitrite formation.

Red wine consumption has been associated with cardio-protective effects and has been proposed as the solution to the French paradox, which questions the relationship between the region's high dietary fat consumption and low incidence of cardiovascular disease. An impairment of endothelium-dependent relaxation is a classic symptom of cardiovascular disease, and is linked to the reduction of bioavailable NO due to vascular formation of superoxide. Increased levels of angiotensin II (Ang II) are also associated with cardiovascular disease. Transgenic hypertensive rats, over-expressing Ang II, demonstrate increased production of O_2^- by way of increased NADPH oxidase expression. The treatment of rats with Ang II dramatically increased the systolic blood pressure and reduced the aortic epitheliums relaxation response to ACh. Pretreatment of rats with red wine polyphenols significantly reduced the effects of Ang II treatment, lowering the blood pressure (Sarr et al. 2006). Additionally, the inhibitory effects of Ang II on ACh induced epithelial relaxion was also countered by consumption of red wine polyphenols. These effects were likely due to decreased expression of NADPH oxidase subunits in polyphenol supplemented rats. Expression of the nox1 and p22phox subunits were decreased in polyphenol and Ang II treated rats versus Ang II treated rats alone.

Oxidized low density lipoprotein (oxLDL) is a critical mediator of pro-inflammatory processes in arterial vascular tissues. Vascular cells which take up oxLDL undergo proapoptotic processes which contribute to atherosclerosis, increase levels of superoxide anion due to NADPH oxidase activation, and exhibit a significant increase in protein oxidative modification and nitration (Steffen et al. 2007) The treatment of bovine aortic epithelial cells with oxLDL produced a down regulation of eNOS expression while inducing iNOS. This contradicts data from other muscle cell types which have shown an inhibition of iNOS due to oxLDL. These effects were countered by pretreatment of the cells with (-)-epicatechin. Whereas oxLDL induced iNOS dependent formation of both nitrite and nitrate, the addition of (-)-epicatechin drives a preferential shift in the formation of nitrite by eNOS. Although initially it would appear that increased NO production via upregulated iNOS would be beneficial, concominant increase in O_2^- production by NOX could easily elevate levels of peroxynitrite and other nitrogen radicals. Epicatechin suppressed the production of nitrate to a greater extent than nitrite, increasing the ratio of nitrite to nitrate, which may be due to a decrease in spontaneously generated nitrate from the reaction of NO and O_2^-. This assertion is supported by the fact that treatment of cells with oxLDL and (-)-epicatechin showed lower oxidative stress than those treated with oxLDL alone. This also resulted in a significant reduction in protein modifications.

Cyclooxygenase is the key enzyme involved in inflammatory responses via conversion of arachidonic acid to prostaglandins, and exists in two isoforms. The COX-1 enzyme is expressed constitutively, while COX-2 is induced by inflammatory stimuli. COX-2 enzyme is expressed in various cancers including colon, lung, and esophagal. The transcription factors NF-κβ, AP-1, and CREB have all been shown to interact with the COX-2 promoter. There is also evidence that LPS, a known potentiator of COX-2, increases COX-2 expression through MAPK signaling, including ERK, JNK, and p38. The treatment of murine macrophage RAE264 cells with LPS was used as a model for assessing the anti-inflammatory properties of anthocyanidins, the aglycones of anthocyanins. Pretreatments with delphinidin or cyanidin effectively suppressed COX-2

induction in LPS treated cells (Hou et al. 2005). Delphinidin inhibited the COX-2 expression at both protein and mRNA levels, indicating transcriptional regulation.

AP-1 exists as a heterodimer of c-Jun and c-Fos. LPS evoked RAW264 cells treated with delphinidin showed near complete inhibition of c-Jun phosphorylation, but no inhibition of CREB was observed. The LPS treatment of RAW264 cells also showed proteolytic degradation of Iκβ protein which is responsible for cytosolic inactivation of NF-κβ. Pretreatment with delphinidin protected the Iκβ from degradation, decreasing pools of active NF-κβ. This was also reflected in decreased nuclear translocation of NF-κβ. Delphinidin also demonstrated inhibition of LPS evoked phosphorylation of all three MAPKs: ERK, JNK, and p38.

Green and black tea polyphenols have also demonstrated inhibitor activity against COX and LOX enzymes in human colon mucosa and colon tumor tissues. Both COX and LOX enzymes are implicated in arachidonic acid metabolism and inflammatory processes, with COX located in microsomes and LOX found predominantly in the cytosol. Catechins of green tea and theaflavins of black tea inhibited both LOX and COX activities (Hong et al. 2001). Enzyme activity was ascertained via quantification of arachidonic acid metabolites. Inhibition of COX enzymes resulted in a greater decrease in the formation of throboxane and 12-hydroxyheptadecatrienoic acid versus other metabolites.

Polyphenolic extracts of red wine and black tea have been shown to inhibit the promotion phase of colon carcinogenesis. Levels of COX-2, iNOS, and glutathione S-transferase (GST) expression were measured in both tumorigenic and normal colon tissues with and without polypheol treatment (Luceri et al. 2002). The colon tumors demonstrated higher levels of all three genes versus normal mucosa. Treatment of tumors with black tea and red wine polyphenols both decreased levels of GST expression, while red wine polyphenols significantly lower expression of COX-2 and iNOS as well.

A vast majority of the credit for the actions of blueberries in *in vitro* models and benefits in *in vivo* models are directed toward the polyphenol component. This is likely due to the prolific body of research on dietary polyphenols. The fact that blueberries are

rich in polyphenolic components make the association of previously described actions of polyphenols and the benefits of dietary blueberry supplementation an easy one to make. Although there is a significant amount of literature supporting the notion that polyphenols possess anti-inflammatory and ant-oxidative activities via modulation of multiple cascades, we saw neither a decrease in nSMase-mediated release of ceramides or inhibition of NOX formation via p67 translocation from any of the prominent blueberry polyphenolic fractions at the concentrations and durations we examined. The catechin and anthocyanidin fractions appeared inflammatory in both assays on the SH-SY5Y neuroblastomas. This does not discredit earlier works on the subject, only refines it. The fact that prominent blueberry polyphenols acted in a pro-inflammatory fashion at particular doses in a particular cell line is likely the results of the biphasic action of anti-oxidative molecules which can demonstrate either protective or deleterious effects depending on the specific cellular environment.

In the context of bioassay-directed fractionation, only one polyphenol was identified with inhibitory properties. DPG has not been previously described in the literature and demonstrated moderated inhibition of both nSMase and NOX under the described experimental conditions. Compared to catechins and anthocyanidins, DPG is a minor secondary metabolite. This is not surprising considering benzoic acid derivatives are far less concentrated in blueberries than catechins or hydroxycinnamic acid derivatives; however, this may be the very reason DPG has not been previously discovered.

4.4 Malic Acid

NADPH is a reducing agent essential to oxidative defense and other biochemical processes. The action of catalase, superoxide dismutase and glutathione peroxidase all depend on cellular store of NADPH for regular function. As much as 60% of NADPH is synthesized in the oxidative phase of the pentose phosphate pathway during normal metabolism; however, in periods of elevated oxidative stress other pathways are utilized

to more rapidly regenerate NADPH. NADPH is also essential for the biosynthesis of lipids.

The action of malic enzyme catalyses the conversion of malate to pyruvate, converting NADP to NADPH in the process. This process has also been shown to interact with elements of gluconeogenesis and the TCA cycle, producing a metabolic network which provides rapid reductive recovery for the cell via the conversion of NADH to NADPH and the rapid recycling of malate (Singh et al. 2008). In this module malate is converted to pyruvate via malic enzyme. Pyruvate is then converted to oxaloacetate via pyruvate carboxylase. Oxaloacetate is then converted to malate via malate dehydrogenase. In periods of oxidative stress, metabolic defenses require sustained levels of NADPH, which is reflected by decreased pools of malic acid. Indeed malic acid appears to be the bottle neck for the rapid production of NADPH. This process is not unique to antioxidant defense. Malic enzyme has also been shown to be involved in lipid accumulation in periods of increased lipid synthesis (Wynn and Ratledge 1997).

Malic acid is rapidly depleted under anaerobic or hypoxic conditions. This can occur during bouts of exercise and can occur locally in fibromyalgia (Russel et al. 1995). Under aerobic conditions malate is converted to oxaloacetate, producing reducing equivalents, but under anaerobic conditions glycolysis is inhibited by the build up of excess reducing equivalents. This is believed to be the source of muscular pain in cases of fibromyalgia. The conversion of malate to succinate is capable of clearing reducing units, so increased pools of malate would appear to improve performance in anaerobic conditions as well as relieve symptoms in fibromyalgia. Supplementation of malic acid in conjunction with Mg^{2+} has shown therapeutic potential in the treatment of fibromyalgia.

Malic acid (5 μg/mL) effectively inhibited TNF-α stimulated NOX activation by 48.4% and inhibited nSMase by 67.9% in SH-SY5Y human neuroblastomas. Mechanistically, these observations make sense. By increasing the cellular supply of malic acid, the bottle neck for the production of NADPH is reduced, making the cell

more capable of dealing with the oxidative insult. Specifically, the regeneration of GSH requires NADPH as a reducing agent, so increased levels of malic acid in periods of oxidative insult will allow for sustained regeneration of GSH. Not only does this make the cell more capable of dealing with redox stress, but GSH acts as an inhibitor of nSMase (Liu et al. 1998). Reduced pools of GSH caused by oxidative insult, leads to the activation of nSMase, which results in increased levels of free ceramides. This results in an inflammatory cascade and activation of NOX. Treating the cells with malic acid likely provided a greater capacity to regenerate GSH, thereby reducing nSMase and NOX activation. This may be of particular importance in Alzheimer's and aging models which have demonstrated increased sensitivity of nSmase and increased levels of free ceramides.

4.5 Blueberric Acid

We have identified blueberric acid as a potent inhibitor of TNF-α-induced potentiation of both NOX and nSMase. Blueberric acid is a significant secondary metabolite in the Alaska bog blueberry, accounting for approximately 5% of the ripe berry's dry weight. Commercially available berries have assayed between 0.5 and 1% BBA by dry weight. Despite its apparent structural similarity to two units of malic acid, blueberric acid is not a dimer of malic acid, and cannot be easily degraded into multiple units of malic acid. Interestingly, blueberric acid proved more potent inhibitor of nSMase than malic acid at equivalent concentrations. The mechanism for the actions of blueberric acid are and the biological role it plays in the berry fruit are unknown, as this is the first time the compound has been described as a natural product.

Due to structural similarities with units of malic acid, it is possible that blueberric acid interacts with enzymes responsible for malic acid metabolism such as malic enzyme. Blueberric acid may be actively metabolized or act as inhibitors by interacting with the substrate-binding surface. If blueberric acid were to act as an inhibitor of malic enzyme, the decreased cellular capacity to produce NADPH might result in an up-regulation of malic enzyme expression. The resulting cellular preconditioning would make the cell

more capable of regenerating NADPH following exposure to blueberric acid. It is also possible that blueberric acid acts as a potent metal chelator. EDTA, is a tetra acid with two nitrogens, which effectively chelates metal cations by forming an octahedral coordinate compound. Blueberric acid may be able to perform the same function via its four carboxylates and two hydroxyl functionalities. This would be a possible explanation for the potentent inhibition of Mg^{2+} dependent nSMase. Multiple avenues are now available for the further study of this natural product involving its absorption, metabolism, and involvement in anti-inflammatory protection.

4.6 Future directions

Methods for rapid quantification of ursolic acid and blueberric acid should be developed. Ursolic acid may be responsible for many of the biochemical properties attributed to polyphenolic compounds in whole food studies. Additionally, the new research demonstrating ursolic acid's reduction in lipid raft formation provides new impetus to quantitate ursolic acid in dietary components associated with anti-inflammatory properties. Being a novel compound, the relative quantities of blueberric acid in food products is open to investigation, especially in foods associated with neuronal function, cardiac function, inflammation control, and insulin response. It would also be important to determine whether or not ursolic acid is incorporated into the outer membrane or is affecting the formation of lipid rafts through interactions in the cytosol.

Developing a synthetic approach to aquiring blueberric acid would be advantageous in subsequent work. This would likely require collaboration with a specialist in synthetic organic chemistry. Having a ready supply of bluebrric acid would be critical in any *in vivo* studies and would also simplify studies on structure and function, providing a ready base for structural modification.

The dose response for each compound should be evaluated to determine relative toxicity in SH-SY5Y neuroblastoma. The concentration at which each compound transitions from providing anti-inflammatory effects versus pro-apoptotic effects could be evaluated via the q-PCR of anti- and pro-apoptotic genes. The specific effects in other

tissue models including respiratory and muscular bares scrutiny as well, especially considering not all observed benefits of whole berry supplementation are neurological. The mechanism for the inhibitory actions of BBA in TNF-α-stimulated nSMase and NOX activation in neuronal cells is open to investigation. It would be compelling to see if BBA interacts with enzymes associated with malic acid metabolism.

It is possible that the ischemic protection of blueberry supplementation is due to an inhibition of the ASIC 1A receptor in the brain. The ASIC 1A is an acid-sensitive Ca^{2+} channel. Its disfunction has been associated with ischemic inury, Alzheimer's disease (which is classically associated with Ca^{2+} misregulation), and learning and memory via LTP function. These are all conditions positively affected by blueberry supplementation. The ASIC 1A receptor function is also affected by insulin. Increased levels of insulin have been shown to reduce trafficking of the receptor from the Golgi to the outer membrane. Polyphenolic components of blueberries and ursolic acid have been impicated in PI3K/Akt response to insulin, and may be associated with the regulation of ASIC 1A transport. The number of aligning variables between recorded ASIC 1A misregulation and benefits of blueberry supplementation makes the initial study of their possible interactions worthwhile.

Reduced symptoms of insulinemia by whole berry supplementation is well established in muscle and adipocyte models as well as *in vivo* studies; however, little work has been done on insulin management in neuronal models. The increased level of free ceramides in neurons has been shown to decrease insulin sensitivity and have been correlated to type III diabetes. Considering that multiple components of the Alaska bog blueberry have been shown to reduce nSMase-mediated ceramide response, it would be valuable to evaluate the insulin management of neuronal cells in conjunction with inflammatory stressors and blueberry supplementation.

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Appendix A

Alaska Wild Blueberry Extracts Inhibit a Magnesium-Dependent Neutral Sphingomyelinase Activity in Neurons Exposed to TNF-α*

A.1 Abstract

The proinflammatory cytokine tumor necrosis factors α (TNF-α) is key to initiating and orchestrating inflammation, which substantially contributes to the progression of many chronic and acute CNS pathologies. TNF-α can stimulate a magnesium (Mg^{2+})-dependent neutral sphingomyelinase (nSMase) resulting in the accumulation of ceramide, a lipid messenger implicated in oxidative stress and apoptosis. Dietary polyphenols were shown to alleviate CNS inflammation largely attributed to their antioxidant properties. We found that preincubation of human SH-SY5Y neuroblastoma cells with aqueous or organic extracts prepared from Alaska wild bog blueberries completely negated Mg^{2+}-nSMase activation upon TNF-α exposure. This specific and potent inhibition of Mg^{2+}-nSMase activity was non-antioxidant in nature. This study demonstrated for the first time that Alaska wild bog blueberries harbor the capacity to interfere with a key step in the progression of inflammation, the activation of Mg^{2+}-nSMase, in neuronal cells further providing evidence for the therapeutic potential of blueberries.

A.2 Introduction

The pro-inflammatory cytokine tumor necrosis factor alpha (TNF-α) exhibits pleiotropic effects in the developing, adult as well as injured nervous system (Merill 1992; Vitkovic et al. 2000; Allan and Rothwell 2001; Viviani et al. 2004). TNF-α affects neuronal survival, contributes to the formation, repair and maintenance of synaptic connectivity, modulates outgrowth of neuronal processes, stimulates gene expression, and

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* Current Topics in Nutraceutical Research, Gustafson SJ, Barth BM, McGill CM, Clausen TP, Kuhn TB, October 2007

regulates neuronal redox-state. TNF-α plays a key role in the initiation and orchestration of inflammatory in many chronic neurodegenerative diseases (Alzheimer's disease, Parkinson's disease, Amyotrophic Lateral Sclerosis), acute CNS injuries (stroke and traumatic injuries), and even psychiatric disorders (autism, schizophrenia) (Fontaine et al. 2002; Vargas et al. 2005; Keane et al. 2006; Lucas et al. 2006). TNF-α is persistently secreted at high levels from reactive microglia and astroglia cells both prevalent in most CNS pathologies as well as in the aging CNS (Block et al. 2007). Interaction of TNF-α with the TNF receptors type I and II activates numerous cellular signal transduction pathways leading to stress kinase activation, oxidative stress, caspase activation, ceramide production, and ultimately apoptosis of neuronal cells (Locksley et al. 2001). Sphingolipids are highly prevalent in neuronal plasma membranes and serve as precursors for various lipid second messengers (Futerman and Hannun 2004). For instance, ceramide is highly cytotoxic for most neuronal cell types and implicated in oxidative stress and apoptosis. Ceramide can be either synthesized *de novo* from the palmitic acid or rapidly accumulates upon hydrolysis of complex sphingolipids by a TNF-α-sensitive magnesium (Mg^{2+})-dependent neutral sphingomyelinase (nSMase). So far, three mammalian Mg^{2+}-nSMase have been characterized where Mg^{2+}-nSMase *2* is specifically expressed in mammalian brain (Clarke et al. 2006). Mg^{2+}-nSMase *2* is implicated in apoptosis, and inflammatory processes yet also in cell growth and differentiation. Inhibiting Mg^{2+}-nSMase *2* activity with GW4869 or via siRNA attenuated TNF-α-stimulated apoptosis and generation of reactive oxygen species. Diets rich in fruit and vegetables were long known for the benefits to our health (Joseph 2002). For instance, dietary intake of blueberries was shown to alleviate cognitive decline in animal models and decreases ischemia-induced brain damage (Mattson et al. 2002; Sweeney et al. 2002; Joseph et al. 2003). The active ingredients are largely attributed to the family of polyphenols, which act *in vitro* as highly effective antioxidants and reduce the progression of CNS inflammation (Ramassamy 2006). However, many polyphenols are barely detectable in the CNS after dietary consumption implying the presence of compounds with non-antioxidant properties serving as specific and potent inhibitors in

adverse inflammatory signaling pathways. We investigated whether Alaska wild bog blueberries (*Vaccinium uliginosum*) have the capacity to interfere with TNF-α-stimulated inflammatory signaling pathways by specifically inhibiting Mg^{2+}-nSMase activity in neuronal cells.

A.3 Materials and Methods

A.3.1 Reagents: Recombinant human TNF-α and an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) Cell Growth assay kit were purchased from Millipore (Temecula, CA). DMEM and Penicillin/Streptomycin solution were obtained from Mediatech (Herndon, VA). GlutaMAX-1, trypsin/EDTA solution, and an Amplex Red Sphingomyelinase assay kits were received from Invitrogen (Carlsbad, CA). Fetal bovine serum was purchased from Atlanta Biologicals (Atlanta, GA). All other reagents were purchased from Sigma (St. Louis, MO).

A.3.2 Cell Culture: SH-SY5Y human neuroblastoma cells were grown in DMEM medium supplemented with 10% fetal bovine serum, 100 U/ml Penicillin and 100 U/ml Streptomycin (humidified atmosphere, 5% CO_2, 37°C) in 100 mm dishes (Falcon). Cells were harvested after incubation with trypsin (0.5 mg/ml)/EDTA (0.2mg/ml), briefly triturated, and plated into 6 well or 96 well tissue culture plates (200,000 cells per well or 60,000 cells per well, respectively). Prior to treatments, SH-SY5Y cells in 6 well pates were grown for 72 h whereas SH-SY5Y cells in 96 well plates were grown for 48 h.

A.3.3 Blueberry Extract Preparation: Alaskan wild bog blueberries (*Vacciunium uliginosum*) were harvested in the interior of Alaska at three distinct geographical locations (Lake Minchumina, Fox, and Fairbanks, Alaska) and stored frozen at -20°C. Blueberries were lyophilized, crushed to a powder, and then extracted without hydrolysis either using an organic solvent or water. To obtain an organic blueberry extract (OBBX), 3.0 g of powdered blueberries were dissolved in 20 ml 70% acetone/30% water for 15 minutes with frequent agitation whereas an aqueous blueberry extract (ABBX) was

prepared using 18 mΩ water. Insoluble material was removed by filtration and extracts were rotovaporized at 40° C (removal of volatile organics) prior to freezing and lyophilizing. The resulting powder form of extracts was stored at -20° C. OBBX or ABBX were reconstituted either in 70% acetone/30% water or 18 mΩ water only followed by a 1:20 dilution with 18 mΩ water immediately before addition to SHSY5Y cells.

A.3.4 Sphingomyelinase Assay: Subconfluent cultures of SH-SY5Y cells were pretreated with 5 μg/ml OBBX or ABBX for 2 h. After exchanging media, SH-SY5Y cells were exposed to TNF-α (100 ng/ml) for 15 min, harvested in cold phosphate buffered saline and sonicated. Quantitative measurement of magnesium-dependent neutral sphingomyelinase activity was performed in cell lysates containing equal amounts of total protein (BCA assay) and using an enzyme coupled Amplex Red fluorescence assay kit (Invitrogen, Carsbad, CA). Briefly, nSMase activity in samples generates ceramide, which is further converted to phosphocholine. Choline is liberated by alkaline phosphatase and then converted to betaine and peroxide by choline oxidase. In last step, horseradish peroxidase (HRP) reacts peroxide with amplex red to the highly fluorescent product resorufin measured at 590 nm using a Beckman Coulter Multimode DTX 880 microplate reader.

A.3.5 Choline Oxidase Assay: Addition of choline to a mixture of choline oxidase, HRP, and Amplex red results in the formation of peroxide and subsequently the fluorescent product resorufin. In the absence of choline, the above mixture was preincubated with OBBX (5 μg/ml), ABBX (5 μg/ml and 75 μg/ml), 5 mM N-acetyl-L-cystein, or 2000 U/ml catalase for 15 min. The reaction was initiated by adding 50 μM choline. After 15 min incubation period (37^0 C), resorufin fluorescence was measured at 590 nm using a Multimode DTX 880 microplate reader.

A.3.6 Cytotoxicity Assay: Briefly, cultures of SH-SY5Y cells were serum deprived overnight and the supplement with blueberry extracts (5 and 75 μg/ml) for 48 h. Cytotoxicity was determined using an MTT cell viability assay according to manufacture's instructions (Chemicon). Formazan generation was measured using a Multimode DTX 880 microplate reader.

A.3.7 Statistical Analysis: Analysis of variance (ANOVA) was used to determine statistically significant differences between treatments ($p>0.05$). Post hoc comparisons of specific treatments were performed using a Scheffe test to determine statistical significance based on the calculated ANOVA data. All error bars represent standard deviations of the mean (STD).

A.4 Results

In various cell types, TNF-α stimulates a magnesium (Mg^{2+})-dependent neutral sphingomyelinase (nSMase) activity, which generates ceramide by hydrolyzing complex plasma membrane sphingolipids, a key step in the progression of inflammation. Dietary consumption of blueberries was shown to alleviate adverse effects of CNS inflammation. Thus, we tested whether organic or aqueous extracts obtained from Alaska wild bog blueberries (OBBX or ABBX, respectively) interfered with TNF-α-stimulated activation of a Mg^{2+}-nSMase in SH-SY5Y human neuroblastoma cells. Mg^{2+}-nSMase activity was quantified utilizing an enzyme assay coupling ceramide generation in cell lysates to Amplex Red fluorescence. As show in Figure 1, SH-SY5Y cells revealed a dramatic increase in Mg^{2+}-nSMase activity (1.72+0.1, n=3, $p<0.05$) upon a 15 min exposure to 100 ng/ml TNF-α compared to control cultures (1.00+0.29, n=3). In contrast, the presence of OBBX or ABBX (5 μg/ml each) negated TNF-α-stimulated activation of Mg^{2+}-nSMase. The most potent inhibition of Mg^{2+}-nSMase activity was achieved with OBBX or ABBX from blueberries collected at Lake Minchumina (LM; 1.14+0.09, n+3, $p<0.05$ and 1.16+0.12, n=3, $p<0.05$, respectively). In comparison, inhibition of Mg^{2+}-nSMase was less pronounced but still significant using OBBX and ABBX prepared from blueberries

collected in Fox (FX; 1.3+0.2, n=3, $p<0.05$ and 1.37+0.22, n=3, $p<0.05$, respectively) or Fairbanks (FB; 1.12+0.18, n=3, $p<0.05$ and 1.31+0.03, n=3, $p<0.05$, respectively). Taken together our findings suggested that Alaska wild bog blueberries contain a potent inhibitor against Mg^{2+}-nSMase activity in human neuronal cells.

Since quantitative measurements of Mg^{2+}-nSMase activity in cell lysates relied on the formation of peroxide, inhibition of Mg^{2+}-nSMase in our experimental paradigm could have resulted from simple peroxide scavenging. Blueberries in general and Alaska blueberries in particular exhibit some of the highest antioxidant capacities measured (Prior 1998). We controlled for this alternative taking advantage of a direct choline oxidation assay. Addition of choline to a mixture of choline oxidase, HRP, and Amplex Red results in the formation of fluorescent resorufin. As shown in Figure 2, neither addition of ABBX nor OBBX to the direct choline oxidation assay interfered with peroxide production at a concentration (5 μg/ml) shown to completely inhibited Mg^{2+}-nSMase activity. However, increasing concentrations by 15 fold (75 μg/ml ABBX) revealed strong peroxide scavenging activity (FX; 66+4% +, n=8, $p<0.05$).
As expected, addition of 5 mM N-acetyl-L-cysteine (51+3%, n=8, $p<0.05$) or 2000 U/ml Catalase (51+2%, n=8, $p<0.05$) to the direct choline oxidation assay abolished peroxide generation compared to 3 μM peroxide, our positive control (42+4%, n=8, $p<0.05$). These findings suggest the non-antioxidant nature of Mg_{2+}-nSMase inhibition in our blueberry extracts.

We further determined viability of human SH-SY5Y neuroblastoma cells upon prolonged exposure to blueberry extracts to verify the specificity of blueberry extracts in inhibiting Mg^{2+}-nSMase activity. Neither OBBX nor ABBX (5 μg/ml each) compromised SHSY5Y cell viability over a 48 h time period compared to control (Fig. 3). Moreover, SHSY5Y cell viability remained at control levels even when increasing concentrations of extracts 15 fold (75 μg/ml ABBX, FX). In contrast, UV irradiation of cells for 20 min drastically reducted cell viability as expected (positive control). This result demonstrates that blueberry extracts (5 μg/ml) did not compromise SH-SY5Y cell viability.

A.4.1 Extracts of Alaska wild bog blueberries inhibit Mg^{2+}-nSMase activity in neuronal cells

Blueberries were harvested at three different locations in the interior of Alaska: Lake Minchumina (LM), Fox (FX), and Fairbanks (FB). Both organic (70% acetone/30% water) and aqueous extracts were prepared (OBBX and ABBX, respectively) as described in materials and methods. Mg_{2+}-nSMase activity was quantitatively measured using an enzyme assay coupled to Amplex Red fluorescence. Exposure of human SHSY5Y neuroblastoma cells to 100 ng/ml TNF-α for 15 min (T) significantly stimulated Mg_{2+}-nSMase activity (filled bar). In contrast, preincubation of SH-SY5Y cells with 5 μg/ml of ABBX (hatched bars) or OBBX (open bars) for 2 h prior prior to TNFα stimulation abolished Mg_{2+}-nSMase activity and was indistinguishable from our control (Con; light gray bar). All values are normalized to control condition. Error bars represent standard deviations of the mean of three independent experiments (duplicates each) and statistical significance was determined at $p<0.05$ (ANOVA and pos hoc Scheffe test).

A.4.2 Inhibition of Mg_{2+}-nSMase activity results from non antioxidant compounds in Alaska wild bog blueberry extracts

Peroxide generation was quantitatively measured via Amplex Red detection in a direct choline oxidation (ChOx). Compared to control (filled bar) neither OBBX nor ABBX exhibited peroxide scavenging capacity at a concentration of 5 μg/ml (open bars and hatched bars, respectively), which were shown to potently inhibit nSMase activity. Yet, addition of 75 μg/ml ABBX to the direct choline oxidation assay exhibited strong antioxidant capacity. As expected 5 mM N-acetyl-L-Cysteine (NAC) and 2000 U/ml catalase (CAT) significantly interfered with peroxide generation. Omitting choline in our assay resulted in virtually no fluorescence (n) and addition of peroxide (H) to Amplex red in the presence of HRP increased fluorescence (positive control). Error bars represent standard deviations of the mean from six independent experiments and statistical significance was determined at $p<0.05$ (ANOVA and post hoc Scheffe test). Extracts

were prepared from Alaska wild bog blueberries harvested at Lake Minchumina (LM), Fox (FX), and Fairbanks (FB).

A.4.3 Extracts of Alaska wild bog blueberries are not cytotoxic

Serum free cultures of SH-SY5Y cells were supplemented with OBBX and ABBX (5 μg/ml each) and maintained for 48 h prior to measuring cell viability (MTT assay). Neither OBBX nor AABX compromised cell viability at concentrations (5 μg/ml) shown to inhibit Mg^{2+}-nSMase activity compared to control conditions. Cell viability remained indistinguishable from control even when increasing ABBX to 75 μg/ml, a 15 fold excess (ABBX-75). However, UV exposure (20 min) of SH-SY5Y cells dramatically reduced cell viability (positive control, UV). Error bars represent standard deviations of the mean of at least four independent experiments and statistical significance was determined at $p<0.05$
(ANOVA and post hoc Scheffe test). We tested blueberries harvested from Lake Minchumina (LM), Fox (FX), and Fairbanks (FB).

A.5 Discussion

We demonstrated for the first time that Alaska wild bog blueberries have the capacity to inhibit a Mg^{2+}-nSMase activity in human neuronal cells upon exposure to the pro-inflammatory cytokine TNF-α (Fig. 1). Moreover, our findings strongly suggested that Mg^{2+}-nSMase inhibition was non-antioxidant in nature (Fig. 2), extractable in either an organic solvent or water only, and benign to viability of neuronal cells over a sustained time period. So far, three distinct Mg^{2+}-nSMase have been characterized in eukaryotes and, relevant to our study, Mg^{2+}-nSMase 2 is predominantly expressed in the mammalian CNS (Hofmann et al. 2000). Although not tested, our studies likely addressed Mg^{2+}-nSMase type 2 activity. First, experiments were performed in cholinergic and dopaminergic SH-SY5Y neuroblastoma cell line derived from a human CNS tumor. Second, this Mg^{2+}-nSMase was stimulated upon exposure of SH-SY5Y cells to TNF-α (Fig. 1) as reported for the mammalian CNS Mg^{2+}-nSMase 2. Third, the

pharmacological compound GW4869 inhibited TNF-α-stimulated activation of Mg^{2+}-nSMase in SH-SY5Y cells (data not shown) in accordance with Luberto et al. (Luberto et al. 2002).

The capacity to inhibit Mg^{2+}-nSMase activity was present in both aqueous and organic extracts of Alaska wild bog blueberries harvested at three distinct locations (Lake Minchumina, Fox, Fairbanks). Although not statistically significant, aqueous or organic extracts prepared from Fox blueberries consistently contained higher potency in inhibiting Mg^{2+}-nSMase compared to either extracts prepared from Fairbanks blueberries of Lake Minchumina blueberries, respectively. These observed differences could arise from extracts not standardized according to a series of phytomarkers or actual distinct compositions of blueberries due to factors such as soil composition, water availability, or sun exposure for despite all three blueberry harvest locations lie within 200 miles of each other in the interior of Alaska. Clearly, future studies need to include a standardized comparison among Alaska blueberries as well as blueberries from other US locations to determine whether significant differences in the potency of inhibiting Mg^{2+}-nSMase activity exist. The health benefits of a high dietary consumption of blueberries and many other fruits and vegetables are largely attributed to polyphenolic compounds with antioxidant properties. Since peroxide production was in integral step in our Mg^{2+}-nSMase activity assay, it was crucial to determine whether our blueberry extracts actively suppressed peroxide production. It is noteworthy that Alaskan bog blueberries contain one of the highest antioxidant capacities measured (Wu 2004). Utilizing a direct choline oxidation assay, we detected no peroxide scavenging in either aqueous or organic blueberry extracts at concentrations of 5 μg/ml irrespective of the harvest location of blueberries. Importantlyat a concentration of 5 μg/ml, Mg^{2+}-nSMase activity was completely blocked with either blueberry extract. Furthermore, aqueous or organic blueberry extracts inhibited Mg^{2+}-nSMase activity in a direct assay using a fluorescent Mg^{2+}-nSMase substrate (data not shown). Nevertheless, addition of aqueous and organic blueberry extracts at 15 fold higher concentrations significantly suppressed peroxide production indicative of their antioxidant properties. Conclusively, inhibition of Mg^{2+}-

nSMase derived from a nonantioxidant compound present in Alaska wild bog blueberries.

Finally, inhibition of Mg^{2+}-nSMase activity could also be account for by an inherent cytotoxic effect of our blueberry extracts on SH-SY5Y cells. Some residual cell death is expected to occur under serum free conditions for 48 h even in controls. Interestingly, both aqueous and organic blueberry extracts showed a strong tendency to increase cell viability compared to our control. Potentially, the high antioxidant properties of blueberry extracts were beneficial for cell viability analogous to B 29 supplementation of cultured hippocampal neurons. Activation of Mg^{2+}-nSMase and subsequent generation of ceramide is key to the progression of inflammation and increased of oxidative stress in the CNS common to many chronic neurodegenerative pathologies, acute CNS injuries, and even psychiatric disorders. Thus, Mg^{2+}-nSMase represents a pivotal target for therapeutic intervention. The fact that Alaska wild bog blueberries contain a potent Mg^{2+}-nSMase inhibitor could further substantiated the benefits of dietary consumption of blueberries to alleviate inflammation in the CNS (Wu et al. 2004; Noyan-Ashraf et al. 2005).

A.6 Acknowledgements

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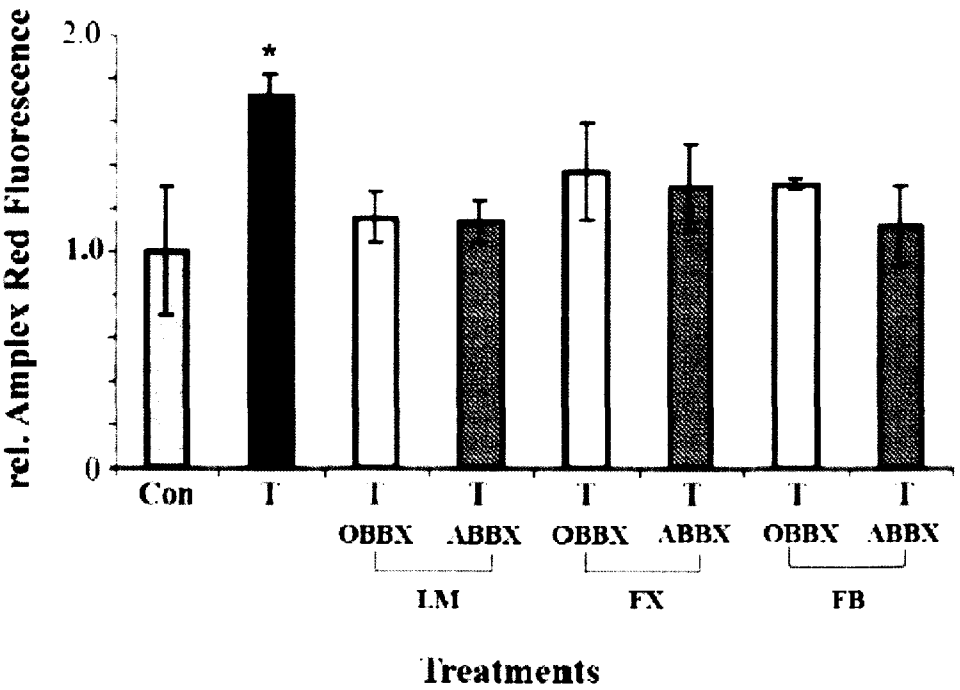

Figure A-1 Extracts of wild Alaskan bog blueberries inhibit Mg^{2+}-nSMase activity in neuronal cells.

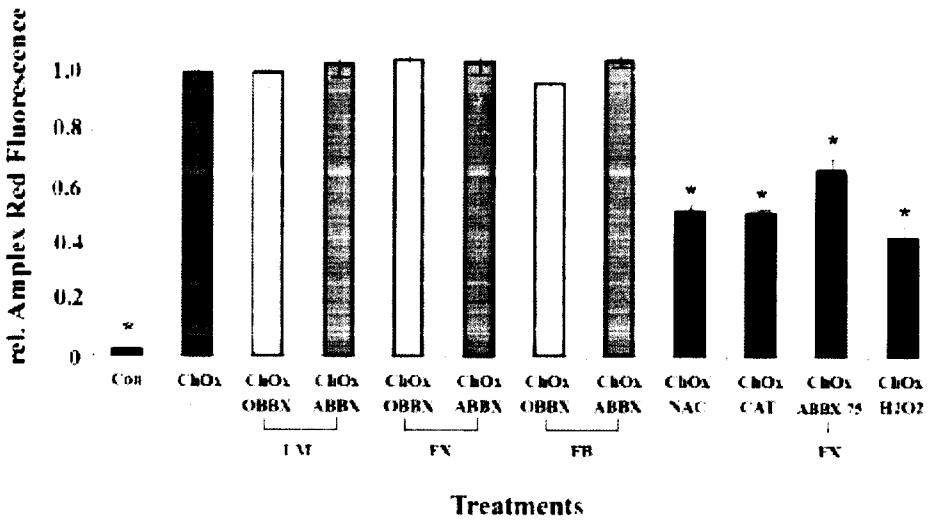

Figure A-2 Inhibition of Mg^{2+}-nSMase activity results from non-antioxidant compounds in wild Alaskan bog blueberry extracts.

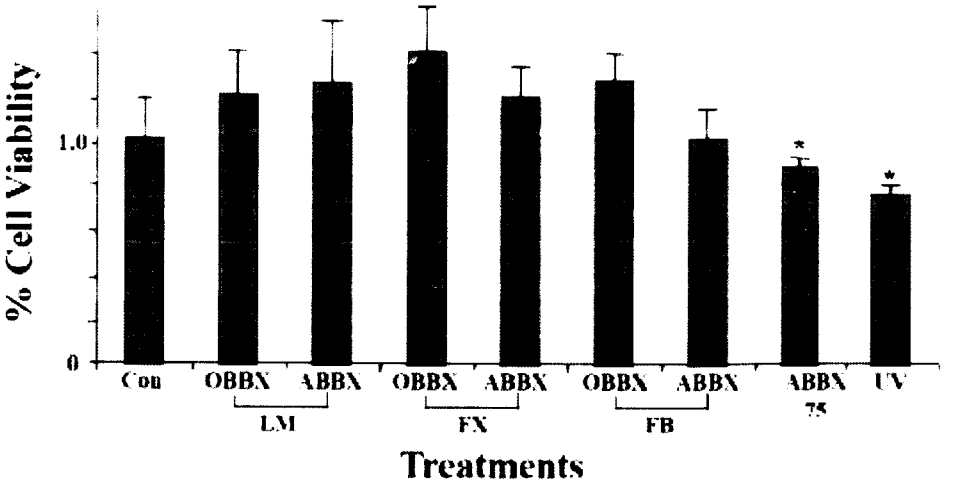

Figure A-3 Extracts of wild Alaskan bog blueberries are not cytotoxic.

Appendix B

A Non-antioxidant Compound Present in Non-polar Blueberry Fraction Inhibits NADPH Oxidase-Mediated Neuroinflammation*

B.1 Abstract

The functional assembly of NADPH Oxidase (NOX) results in production of the reactive oxygen species, superoxide (Bedard et al. 2007; Heyworth et al. 2003). Accumulation of ROS in the central nervous system (CNS) increases neuronal stress and oxidative damage that accompany NOX related neuroinflammation (Lambeth et al. 2008). Established neuroinflammatory pathways are prevalent to those with neuronal injury and acute, chronic, or psychiatric conditions. Molecular compounds found in fruits and vegetables are of interest as potential inhibitors for key enzymes that influence neuroinflammatory pathways. Here, we report that a non-antioxidant component present in non-polar wild Alaskan blueberry fractions inhibit the translocation of the NOX cytosolic subunit, $p67^{phox}$, to the plasma membrane in neuroblastoma cells. We demonstrate that the mode in which this translocation is inhibited is by modulation of lipid raft (LR) signaling platforms in neuroblastoma cells. These findings provide evidence for the therapeutic potential of blueberries in inhibiting NOX assembly and ultimately in decreasing superoxide formation and the progression of CNS inflammation.

B.2 Introduction

The NADPH Oxidase (NOX) enzyme consists of two integral membrane proteins, $gp91^{phox}$ and $p22^{phox}$, otherwise known as the flavocytochrome b_{558} (cyt b_{558}) (Lin et al. 2007), and three cytosolic subunits, $p67^{phox}$, $p47^{phox}$, and $p40^{phox}$ (Bedard et al. 2007). NOX activation is orchestrated through a series of protein-protein interactions that lead to the translocation of the cytosolic subunits to the membrane where they interact with

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$p22^{phox}$ of the cyt b_{558}. This translocation is initiated by conformational changes of $p47^{phox}$ via phosphorylation. The functional assembly of NOX is pertinent for the innate immune response known as phagocytosis, which serves as a host defense mechanism against microbial infection by producing superoxide (Stuart et al. 2005). While basal levels of NOX-mediated ROS are beneficial to the human biological system, prolonged NOX activity possesses less profitable inflammatory effects. The CNS is extremely susceptible to the hazardous consequences of oxidative damage (Olanow 1993) and inflammation associated with NOX-mediated ROS. NOX isoforms are established throughout mammalian tissue and play significant roles in the onset and progression of many diseases. NOX participation is evident in general aging, chronic granulomatous disease, hyperthyroidism, cardiovascular physiology, vascular ailments including hypertension, and a plethora of neurodegenerative diseases including Amyotrophic Lateral Sclerosis (ALS), Neimann-Pick disease, Alzheimer's, Parkinson's, and other forms of dementia (Vigone et al. 2005) (Harraz et al. 2008).

Inflammatory mediators are key to the progression of neurodegeneration in CNS pathologies. The pro-inflammatory cytokine tumor necrosis factor-α (TNF-α) is released by activated Microglia and stimulates Mg^{2+}-dependent neutral sphingomyelinase (nSMase) activity. This activity rapidly generates ceramide via hydrolysis of sphingomyelin (Schematic 1) and is prevalent in neuronal plasma membranes. The bioactive lipid messenger ceramide is implicated in oxidative stress, by means of NOX, as well as apoptosis in CNS neurons (Cutler et al. 2004; Ichi et al. 2009). Alternatively, phorbol 12-myristate-13-acetate (PMA) directly (Figure 3.1) stimulates the assembly of NOX. PMA activates protein kinase C (PKC), which results in the phosphorylation of $p47^{phox}$. This phosphorylation is crucial to the functional assembly of NOX.

Foods containing nutritional antioxidants have long been considered to decrease the effects of oxidative stress and inflammation coupled to neurodegenerative diseases (Engelhart et al. 2002; Galli et al. 2002; Joseph et al. 2005). The beneficial properties of berries have been related to their bioactive phytochemicals, including polyphenols, sillbenoids, and triterpenoids (Seeram 2008). The vast nutritional components that

blueberries are known to possess have even been found to have anti-aging effects and to decrease levels of pro-inflammatory cytokines associated with neuroinflammation (Paul et al. 2010). Understanding the mechanism of NOX inhibitors may lead to a better understanding of this elaborate membrane bound enzyme and give insight to development of therapeutics for decreasing or preventing inflammation of the CNS. Here, we investigated the effects of a non-antioxidant component, present in non-polar blueberry fraction (NP_{BB}), on NOX in neuroblastoma cells.

B.3 Materials and Methods

B.3.1 Reagents

DMEM and Penicillin/Streptomycin solution were obtained from Mediatech (Herndon, VA). GlutaMAX-1, trypsin/EDTA solution, TMB (3,3',5,5'-tetramethylbenzidine), H_2DCFDA (2',7'-dihydrodicholorfluorecein diacetate), Alexa fluor 555 Lipid Raft labeling kit and NuPAGE running and transfer buffers were purchased from Invitrogen (Carlsbad, CA). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) Cell Growth assay kit was obtained from Millipore (Temecula, CA). Anti- $p67^{phox}$ polyclonal antibody and goat anti-rabbit IgG secondary antibody conjugated to HRP were from Abcam (Cambridge, MA). BCA Protein assay kit and 1-step NBT/BCIP were from Pierce (Rockford, IL), PMA from Biomol (Plymouth, PA), and fetal bovine serum was received from Atlanta Biologicals (Atlanta, GA). Recombinant human TNF-α was received from ProSpec (Rehovot, Israel). All other reagents were purchased from Sigma Aldrich (St. Louis, MO).

B.3.2 Cell Culture

SH-SY5Y human neuroblastoma cells were grown in DMEM medium supplemented with 10% fetal bovine serum, 100 U/ml Penicillin, 100 U/ml Streptomycin, and 1% GlutaMax-1, (humidified atmosphere, 5% CO_2, 37°C) in 100 mm dishes (Falcon). Cells were harvested after incubation with trypsin (0.5 mg/ml)/EDTA (0.2 mg/ml), briefly triturated, and were plated into poly-D-lysine coated glass cover slips in

35 mm dishes (confocal microscopy) or into tissue culture treated 6 well or 96 well plates. Cells were grown for 24 hours to 40% (confocal microscopy) or 80% confluency and were then serum starved overnight in DMEM, 1% GlutaMax-1, 100 U/ml penicillin and 100 U/ml streptomycin (humidified atmosphere, 5% CO_2, 37°C) prior to experimentation.

B.3.3 Blueberry Extract Preparation

Blueberries (Vacciunium uliginosum) were harvested and stored frozen (-20°C). Blueberries were lyophilized and then crushed to a powder. Approximately 20 grams of sample was fractionated over a silica column in 500 ml segments with a mobile phase of 80/20 dichloromethane/methanol. Extracts were filtered, roto-vaporized at 40°C to remove volatile organics, frozen and then lyophilized again. The powder extracts were stored at -20°C until reconstituted immediately prior to use in a 70/30 acetone/water solution, and diluted 1:20 into deionized (18 mega Ω) water as our stock solutions of non-polar and polar blueberry fractions.

B.3.4 Quantification of Reactive Oxygen Species Production

Cells were grown in 6 well tissue culture plates and serum deprived overnight. 1 hour pre-treatments of DPI (10 uM), NAC (1 mM), AEBSF (1 mM), GW (13.8 uM), and 5 ug/ml of non-polar and polar blueberry fractions were administered in conjunction with H_2DCFDA (1uM). Media was removed and replaced with new serum free media containing TNF-α (200 ng/ml), PMA (400 ng/ml), or no stimuli (control, nt) for 1 hour – during which H_2DCFDA is oxidized to DCF by peroxide. Cells were washed with 1X PBS, lysed (2 M Tris-Cl pH 8.0, 2% SDS, 10 nM Na_3VO_4) and transferred to a black falcon dish. ROS formation was quantified as DCF-fluorescence intensity using a Beckman Coulter Multimode DTX 880 microplate reader (495 nm excitation filter, 525 emission filter).

B.3.5 Choline Oxidase Assay

Addition of choline to a mixture of choline oxidase, HRP, and amplex red results in the formation of peroxide and consequently increases the fluorescent product resorufin. To assay the interference of peroxide production, 5 μg/ml and 75 ug/ml of non-polar (NP_{BB}) and polar (PO_{BB}) fractions or catalase (2000 U/ml) was incubated with 1X reaction buffer for 1 h. The above mixture was then added to the preincubated treatments for 15 mins, prior to addition of 50 μM choline for another 15 mins at 37° C. Resorufin fluorescence was measured at 590 nm using a Beckman Coulter Multimode DTX 880 microplate reader.

B.3.6 Cytotoxicity Assay

Briefly, cultures of SH-SY5Y cells were serum deprived overnight followed by treatments of Milli-Q H_2O, and 5, 30, or 75 ug/ml blueberry extracts (non-polar and polar) for 48 hours. Potential toxic effects on cell viability were monitored using an MTT assay according to manufacture's instructions (Millipore) and formazan generation was measure using a Beckman Coulter Multimode DTX 880 microplate reader.

B.3.7 ELISA Assay for $p67^{phox}$

Cells were grown in 100 mm dishes to confluency and serum deprived overnight followed by 1 hour pre-treatments of 5 ug/ml non-polar or polar blueberry fractions. Cells were then stimulated with TNF-α (200 ng/ml) or PMA (400 ng/ml) for 1 hour and harvested. A cellular fractionation was performed to obtain cytosolic and plasma membrane fractions. Protein quantification of the membrane fractions were determined using a BCA assay (Pierce) and 20 ug/ml of protein in 1X TBST was plated to a 96 well high protein absorbent plate (Falcon) for 12 hours at 25°C. Wells were blocked with 5% w/v BSA in 1X TBST (1 h), and incubated with $p67^{phox}$ primary antibody (3 ug/ml, 4°C, overnight). Next, the cells were rinsed with 1X TBST and incubated with corresponding secondary antibody (1:2000, 45 mins, 25°C) prior to addition of tetramethylbenzidine

(TMB) (100 ul/well, 10 mins). A colorimetric analysis was performed using a Beckman Coulter Multimode DTX 880 microplate reader to measure the absorbance at 620 nm.

B.3.8 Gel Electrophoresis and Western Blotting

For SDS-polyacrylamide gel electrophoresis, equal amounts of total protein (5 μg total membrane protein) were loaded on a 10% agarose gel and separated (125 V, 50 W, 40 mA) using NuPAGE MES running buffer (Invitrogen). Proteins were transferred to nitrocellulose membranes (100 V, 350 mA, 50 W, 1 hr) using NuPAGE transfer buffer (Invitrogen) and then blocked 1 hr with 5% w/v BSA in 1X TBST. Membranes were incubated overnight with primary antibody (1:500 in 1X TBST, anti-$p67^{phox}$, 4° C), washed (1X TBST), and then incubated with the corresponding secondary antibody (1:5000 in 1X TBST, 1 hr, room temp) conjugated to alkaline phosphatase. Immunoreactivity was detected by colorimetric detection using NBT/BCIP (Pierce).

B.3.9 Confocal Microscopy

Human SH-SY5Y neuroblastoma cells were grown on poly-lysine coated glass cover slips (0.13 mm thick German glass) in medium for 24 h. Cells were serum-starved overnight and incubated 1 h with non-polar or polar blueberry fractions (5 ug/ml) prior to acute addition of TNFα (200 ng/ml) or PMA (400 ng/ml). Cultures were labeled with Alexa fluor Lipid Raft Labeling kit 555 (Invitrogen) according to manufactures instructions. Cultures were fixed for 20 mins with 4% paraformaldehyde at 4°C, permeabalized for 20 mins with 0.3% TritonX-100 in 1X PBS at 4° C, and then blocked with 1% normal goat serum in 1X PBS. Cultures were incubated with primary antibody (anti-$p67^{phox}$, 1:200, overnight, 4°C) in 0.1% TX-PBS. Cells were washed (1X PBS) and incubated with secondary FITC-conjugated goat-anti-rabbit antibody (1:200, 2 h, RT, light protected). Finally, cultures were rinsed with 1X TBS and cover slips were mounted with PVA-DABCO. After drying overnight at room temperature, slides were stored at 4°C until image analysis. Images were acquired (63x, oil, Plan Fluor) with a Zeiss confocal microscope LSM 510 equipped with a He/Ne laser and an Argon laser using

filter combinations for Alexa 555 (554 excitation, 570 emission) and FITC (494 excitation, 517 emission). Zeiss LSM Software was used for image acquisition and analysis. For each treatment condition, random fields of view were analyzed and 3 cells from two independent sets of experiments ($n = 6$) were examined for the presence of lipid raft platforms (red), $p67^{phox}$ (green) membrane translocation, and colocalization (yellow).

B.3.10 Cellular Fractionation

Cells were grown to confluency, treated, harvested and sonicated in a sucrose buffer (20 mM Tris-HCL, 2 mM EDTA, 0.5 EGTA, 2 mM AEBSF, 25 ug/ml Leupeptin, 0.33 M Sucrose, pH 8.0). Suspensions were centrifuged at 14000 rpm for 15 mins (Labnet spectrafuge 16M) and the supernatant was removed (cytosolic fraction). The pellet was then resuspended and sonicated in a buffer containing 20 mM Tris-HCL, 2 mM EDTA, 0.5 mM EGTA, and 2 mM AEBSF, pH 8.0. Cells were centrifuged again for 15 mins and the supernatant was collected (plasma membrane). A micro BCA protein analysis (pierce) was used according to manufacturers instructions to determine the protein concentration for each sample.

B.3.11 Statistical Analysis

Data are presented as means±standard deviations except for data presented for figure 5 which are presented as means±standard error of the mean. Significant differences between treatments and within multiple groups ($p<0.05$) were examined using analysis of variance (ANOVA) followed by Tukey's Post hoc analyses performed using Statistical Analysis System (SAS) software.

B.4 Results

B.4.1 Alaskan wild Blueberry Fractions Contain Compounds That Inhibit the Functional Assembly of NOX in Response to Inflammatory Cytokines in Neuronal Cells

The underlying mechanistic characteristics of identified NOX inhibitors are either unspecific or unknown, leaving NOX functionality and its implications in a vast array of diseases inadequately understood. The proinflammatory cytokine TNF-α stimulates NOX via an upstream mechanism that generates ceramide as a key step in the progression of inflammation while PMA assists in $p47^{phox}$ phosphorylation and ultimately in direct NOX activation. It is important when identifying a NOX inhibitor to look at various forms of NOX activation to help conclude the mechanism in which the inhibitor is acting.

B.4.2 Non-polar Blueberry Fraction Inhibits Oxidative Stress of Neuroblastoma Cells Exposed to TNF-α and PMA

CNS inflammation accompanied by oxidative stress is prevalent in normal aging and in many chronic neurodegenerative disorders, including Alzheimer disease (Apelt et al. 2004; Armitage et al. 2009). Recent findings implicate members of the NOX family as pivotal sources of oxidative stress (Armitage et al. 2009). Joseph et a. (2010) demonstrated that nutritional supplementation with blueberry reduces aging associated deficits, such as oxidative stress, in the CNS (Krikorian et al.). We explored whether blueberry supplementation would harbor the capacity to inhibit NOX dependent ROS formation in neuronal cells exposed to TNF-α or PMA. In this study SH-SY5Y human neuroblastoma cells, loaded with DCF, were incubated with pharmacological inhibitors or blueberry fractions from extracts, prior to exposure of TNF-α or PMA. Crude wild Alaskan blueberry extract inhibited TNF-α mediated ROS formation, compared to control (C) (inset, Figure 1A). Importantly, a non-polar fraction obtained from Alaskan blueberry extract was sufficient to completely inhibit ROS formation in SH-SY5Y cells exposed to TNF-α (1.03 ± 0.04, n=4), compared to control (0.99 ± 0.07, n=4) and in contrast to a polar blueberry fraction obtained from Alaskan blueberry extract (2.53 ± 0.1,

n=4) (Figure 1A). In the absence of blueberry fractions, TNF-α strongly stimulated ROS formation (1.91 ± 0.06, n=4). In contrast, polar blueberry fraction was ineffective in inhibiting NOX (10um DPI: 1.07 ± 0.06, n=4; 1mM AEBSF: 0.99 + 0.04, n=4), scavenging ROS (1mM NAC: 0.9 ± 0.06, n=4) or blocking nSMAse activity (13.8uM GW4869: 0.68 ± 0.26, n=4) all of which negated TNF-α stimulated ROS formation. Since PMA is a well-documented strong activator of NOX we tested whether NP_{BB} would also inhibit PMA stimulated ROS formation in neuronal cells (Figure 1B). As expected exposure of SH-SY5Y cells to 400 ng/ml PMA resulted in ROS formation (1.93 ± 0.30, n=4), compared to control (1.00 ± 0.08, n=4). Both 10 uM DPI and 1 mM AEBSF (0.84 ± 0.16, n=4 and 0.52 ± 0.13, n=4) as well as 1 mM NAC (0.96 ± 0.22, n=4) all abolish PMA stimulated ROS formation. Indeed, NP_{BB} also inhibited PMA stimulated ROS formation (1.07 ± 0.16, n=4) whereas PO_{BB} was ineffective (1.85 ± 0.25, n=4). Taken together, the potency of Alaska blueberries to inhibit NOX dependent ROS formation in neuronal cells, exposed to TNF-α or PMA, partition exclusively into a non-polar blueberry fraction.

B.4.3 Non-polar Blueberry Fractions Lack ROS Scavenging Capacity

Blueberries are known to have exceptionally high antioxidant capacity measured as ORAC levels (Wang et al. 2008). Since quantitative measurements of ROS rely on the oxidation of fluorescent indicator DCF by peroxide it was necessary to examine antioxidant capacity of the blueberry fractions. We employed a choline oxidation assay to determine the ROS scavenging properties of non-polar and polar blueberry fractions (Gustafson 2007). As shown in Figure 2, NP_{BB} fraction both at 5 or 75 ug/ml exhibited no ROS scavenging capacity indicated by the complete lack of interference with peroxide formation (2.92 ± 0.27, n=5 and 3.16 ± 0.25, n=5), compared to control without choline (C, 1.00 ± 0.07, n=5) or addition of catalase (0.98 ± 0.04, n=5). In the absence of blueberry fractions or catalase the addition of choline generated formation of fluorescent resorufin indicating peroxide production (4.08 ± 0.05, n=5). However, PO_{BB} fraction revealed ROS scavenging capacity at 75 ug/ml (1.9 ± 0.25, $^{**}p<0.05$), compared to NP_{BB}

(75 ug/ml), which was not observed at 5 ug/ml PO_{BB} fraction (3.08 ± 0.15, n=5). Notably, neither non-polar or polar blueberry fraction compromised neuronal viability at concentration from 5 ug/ml as high as 75 ug/ml (Figure 3). In summary the potency of NP_{BB} fraction to inhibit NOX dependent ROS formation was not the result of an ROS scavenging capacity or reduced neuronal viability and hence suggesting a potential interference with the function assembly of NOX.

B.4.4 Non-polar Blueberry Fractions Interfere with Functional NOX assembly in Plasma Membrane

NOX2 is comprised of $gp91^{phox}$ and $p22^{phox}$ membrane subunits, and the three cytosolic subunits $p67^{phox}$, $p40^{phox}$, and $p47^{phox}$ (Bedard et al. 2007). Generation of ROS by NOX requires functional assembly of cytosolic and membrane subunits. The degree of $p67^{phox}$ translocation from the cytosol to the plasma membrane is a widely accepted indication of functional NOX assembly. We investigated whether NP_{BB} fraction would interfere with NOX assembly using an ELISA assay directed against $p67^{phox}$. As shown in Figure 4A, exposure of SH-SY5Y cells to 200 ng/ml of TNF-α for 30 minutes caused a significant accumulation of $p67^{phox}$ in the plasma membrane (1.60 ± 0.28, n=4), compared to control (1.00 ± 0.07, n=4). Most importantly, NP_{BB} completely abolished $p67^{phox}$ accumulation in plasma membrane (0.92 ± 0.06, n=4) as opposed to PO_{BB} fractions (1.8 ± 0.06, n=4). Utilizing the same experimental design, we directly stimulated functional NOX assembly using PMA (2.10 ± 0.2, n=8), compared to control (1.00 ± 0.11, n=8) (Figure 4B). As determined with TNF-α as a stimulus, NP_{BB} (5 ug/ml) also negated PMA mediated translocation of $p67^{phox}$ to plasma membrane (1.14 ± 0.14, n=8) in contrast to PO_{BB}, which was ineffective (2.00 ± 0.21, n=8). Next, SH-SY5Y cells were exposed to TNF-α (200 ng/ml) or PMA (400 ng/ml) in the presence or absence of non-polar (NP_{BB}) and polar (PO_{BB}) blueberry fractions. Translocation of $p67^{phox}$ to the plasma membrane was analyzed by western blotting and by quantitative densitometry (Figure 5). As expected 200 ng/ml TNF-α or 400 ng/ml PMA stimulated translocation of $p67^{phox}$ into plasma membrane (4.77 ± 0.74, 2.80 ± 0.50, n=3). The effects of blueberry

fractions on translocation of $p67^{phox}$ to the plasma membrane was corroborated with NP_{BB} fraction abolishing functional NOX assembly in the presence of TNF-α and PMA (1.07 ± 0.23, 1.07 ± 0.14, n=3). PO_{BB} fraction was ineffective at inhibiting $p67^{phox}$ translocation when induced by TNF-α (3.3 ± 0.65, n=3) or PMA (3.4 ± 0.12, n=3).

B.4.5 Functional Assembly of NOX is Associated with Lipid Raft Platforms

Lipid raft microdomains exist as organized structures for signaling pathways (Brown 2002) and recent evidence shows that functional NOX assembly is dependent on the formation of these signaling platforms. We investigated whether non-polar (NP_{BB}) and polar (PO_{BB}) blueberry fractions were capable of modulating the formations of lipid raft (LR) platforms using immunocytochemistry followed by confocal microscopy (Figure 6). Here we demonstrate that SH-SY5Y cells exposed to NP_{BB} (Figure 6B) resulted in minimal raft formations as well as nominal $p67^{phox}$ translocation and colocalization when compared with control – no pre-treatments (No PT, Figure 6A). Exposure to PO_{BB} (Figure 6C) fraction under the same conditions resulted in few LR formations, $p67^{phox}$ translocation to the membrane and colocalization of LR and $p67^{phox}$ compared to control (Figure 6A). As expected, SH-SY5Y cells stimulated with PMA (Figure 6D) resulted in translocation of $p67^{phox}$ to the cellular membrane and discontinuous LR and colocalization puncta compared to cells treated with TNF-α (Figure 6G), which resulted in $p67^{phox}$ translocation, inclusive and continuous LR formations, and colocalization of $p67^{phox}$ and LR. PO_{BB} pre-treated cells exposed to PMA (Figure 6F) show significant $p67^{phox}$ translocation as well as LR and colocalization congruent with that of PMA (Figure 6D) treated cells. As expected, TNF-α induced continuous lipid raft formation, $p67^{phox}$ translocation to the plasma membrane, and colocalization of $p67^{phox}$ and lipid rafts (Figure 6G) compared to NO PT (no pre-treatment, control, A). More importantly, we show that cells pre-treated with NP_{BB} prior to TNF-α exposure (Figure 6H) have modulated LR platforms and discontinuous colocalization in addition to minimal $p67^{phox}$ translocation to the plasma membrane. PO_{BB} was ineffective in modulating TNF-α induced LR platforms and $p67^{phox}$ translocation

(Figure 6I). These results support much of the data presented in Figure 5 in addition to suggesting the inhibition of $p67^{phox}$ translocation by non-polar blueberry fraction (NP_{BB}) coincides with a modulation of LR platforms that it turn decreases NOX-mediated ROS.

B.5 Discussion

We demonstrated for the first time that NP_{BB} fractions of Wild Alaskan Blueberries contain the ability to inhibit NOX-mediated ROS in neuroblastoma cells both directly and indirectly stimulated (See Schematic 1). Additionally, our findings reveal that the mechanism in which NP_{BB} inhibits NOX-mediated ROS is not linked to the antioxidant properties associated with that of blueberries. In fact, we show that NP_{BB} is non-antioxidant in nature (Figure 2) even at concentrations that are fifteen-fold (75 ug/ml) what we use as our inhibitory concentration (5 ug/ml). Moreover, our findings suggest that the mode of inhibition established by NP_{BB} is through the prevention of $p67^{phox}$ translocation from the cellular cytosol to the plasma membrane (Figure 4). We show that NP_{BB} enables the prevention of this crucial translocation in the presence of both TNF-α and PMA (Figure 4A, 4B, respectively) and thus inhibits the functional assembly of NOX. The ability of NP_{BB} to inhibit the function assembly of NOX in turn decreases NOX-mediated ROS production in stimulated neuroblastoma cells (Figure 1). To account for decreased NOX activity associated with possible cytotoxic effects of both blueberry fractions used in our experimentation, we measured the cell viability of SH-SY5Y cells treated with NP_{BB} and PO_{BB} (Figure 3). Neither fraction showed compromised cell viability effects at our standard concentration (5 ug/ml) or up to fifteen-fold this concentration (75 ug/ml). Western blot analysis (Figure 5) shows that both TNF-α and PMA increase relative intensity of $p67^{phox}$ in plasma membrane fractions compared to untreated SH-SY5Y cells. Furthermore, we demonstrate that NP_{BB} decreases the relative intensity of $p67^{phox}$ in plasma membrane in the presence of both TNF-α and PMA while PO_{BB} does not have this same effect. It is known that the proinflammatory cytokine, TNF-α, is a strong activator of NOX-mediated ROS formation in neuronal cells (Barth et al. 2009) and research shows that TNF-α is also instrumental in the formation

of lipid rafts (Doan et al. 2004). Using immunocytochemistry and confocal microscopy we assessed the effects of NP_{BB} and PO_{BB} on lipid raft formation (Figure 6). Here we verify the results of Figure 5 using cellular imaging and in addition show that NP_{BB} modulates LR platforms. In conclusion, the mode of NOX inhibition demonstrated by NP_{BB} may be due to the modulation of LR platforms and in turn the prevention of $p67^{phox}$ to the plasma membrane.

Our results contribute to the usefulness of a dietary regimen high in fruits and vegetables; a concept that has long been known to yield health benefits. In particular, the high levels of polyphenolic compounds found in blueberries attribute to decreased ROS and CNS inflammation, alleviation of cognitive decline in animal models, reduced ischemia-induced brain damage (Joseph et al. 2003; Sweeney et al. 2002), and even improved memory in humans (Krikorian et al.). In addition, Wild Alaskan Blueberry extracts with no detectable antioxidant capacity have been shows to inhibit neutral sphingomyelinase activity in neurons exposed to TNF-α (Gustafson 2007). This phenomenon, along with our results, exploits the idea of nutritional intervention and natural product oriented drug development. Our results demonstrate that components other than antioxidants, found in blueberries, may also yield profitable health benefits when incorporated into a diet.

The NOX enzyme is linked to a vast array of diseases that are primarily associated with inflammation and aging. More specifically, NOX is attributed to increased oxidative stress in the CNS common to many chronic neurodegenerative pathologies (Bedard et al. 2007). Determination of specific NOX inhibitors is critical for the development of potential therapeutics to combat the detrimental effects linked to NOX-mediated ROS. While NOX inhibitors remain scarce, this study gives hope to nutritional intervention and disease prevention through diet. The paradigm presented here provides evidence of a non-antioxidant compound present in non-polar blueberry extract that inhibits NOX-mediated ROS and validates the efficacy of dietary constituents, other than antioxidants.

B.6 Acknowledgements

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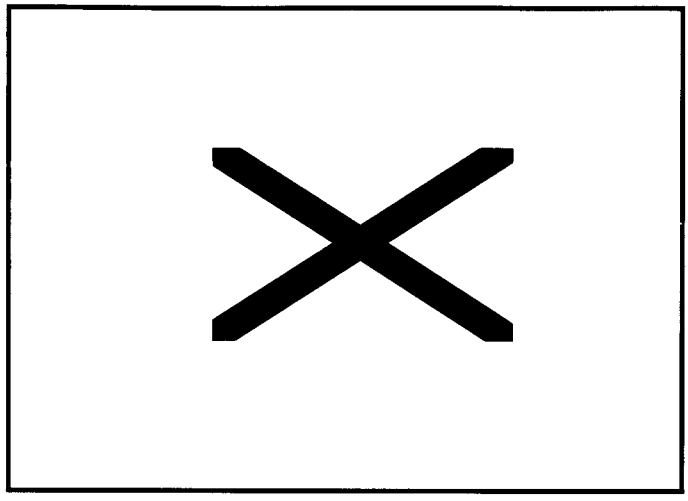
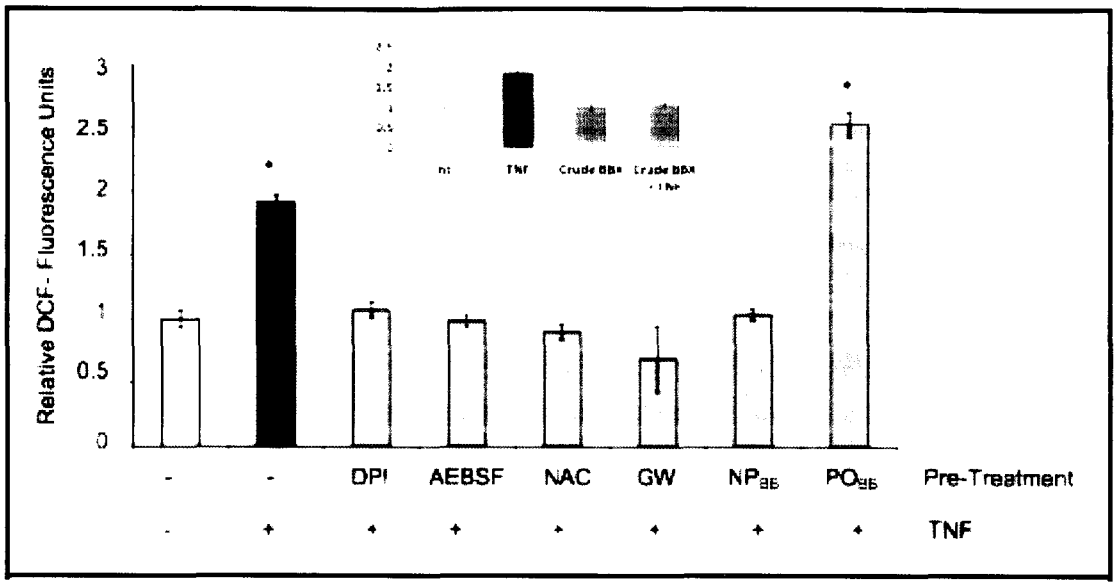


Figure B-1 Activation of NADPH Oxidase. TNF-α indirectly stimulates NADPH Oxidase by liberating ceramide from sphingomyelin in a signaling cascade that increases lipid raft platform formation and interactions with the cellular cytoskeleton to allow the translocation of the NADPH Oxidase cytosolic subunits to the membrane subunits so that the enzyme can become fully functional. Another source of NADPH Oxidase activation occurs in the presence of PMA, which is known to increase phosphorylation of a specific NADPH Oxidase cytosolic subunits and in turn activate the oxidase in a more direct fashion.

A



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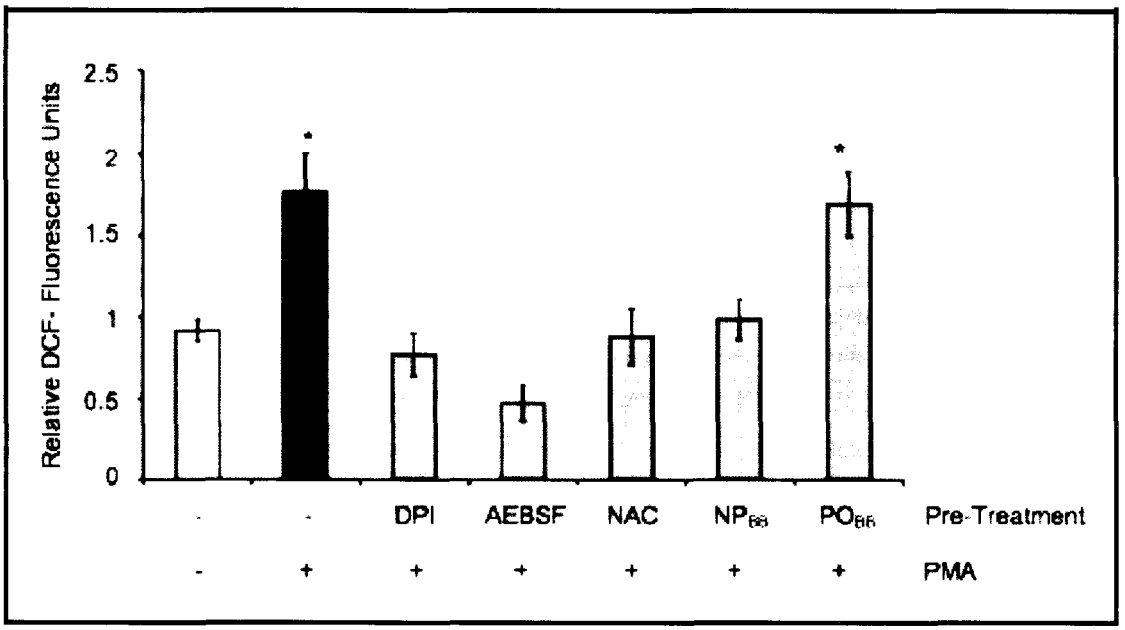


Figure B-2 Non-polar blueberry fraction inhibits oxidative stress in neuroblastoma cells exposed to TNFα and PMA. SH-SY5Y human neuroblastoma cells were loaded for 1 h with 50 uM 2',7'-dihydrodicholorfluorecein diacetate (H_2DCFDA) in the presence or absence of pharmacological inhibitors or non-polar (NP_{BB}) and polar (PO_{BB}) blueberry fractions. Next, cultures were exposed for 1 h to 200 ng/ml TNF-α or 400 ng/ml PMA

and ROS formation was quantified as DCF-fluorescence intensity of whole cell lysates. All measurements were normalized to the average maximum fluorescence intensity under control conditions. (A) TNF-α exposure of SH-SY5Y cells resulted in a significant increase in ROS formation (filled bar), which was negated by the presence of 10 uM DPI, 1 mM AEBSF, 1 mM NAC, 13.8 uM GW4869 (gray bars), or non-polar blueberry fraction (NP_{BB}), compared to control (open bar). Polar blueberry fraction (PO_{BB}) was ineffective in inhibiting ROS formation compared to control (open bar). (B) PMA exposure of SH-SY5Y cells also resulted in a significant increase in relative maximum DCF-fluorescence (filled bar), which was abolished in the presence of NOX inhibitors (10 uM DPI, 1 mM AEBSF), antioxidants (5 mM NAC) (gray bars), or non-polar blueberry fraction (NP_{BB}) compared to control (open bar). PMA evoked ROS formation was not effected in the presence of polar blueberry fraction (PO_{BB}), compared to control (open bar). All data represent the mean of at least four independent experiments ± standard deviations (SD), and statistical significance was determined at $*p<0.05$ (ANOVA and Tukey's *post hoc* analysis).

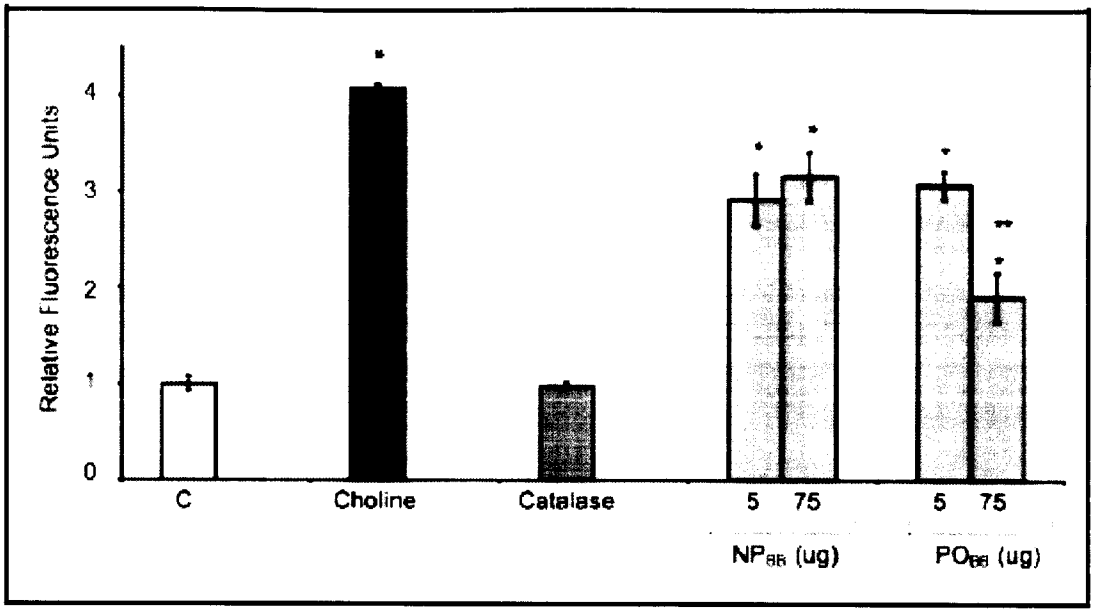


Figure B-3 Non-polar blueberry fractions lack ROS scavenging capacity. Peroxide generation was quantified via Amplex Red in a direct choline oxidation assay through the conversion of choline to betaine and peroxide via choline oxidase. All fluorescence measurements were normalized to omission of choline, our control (C, open bar). Addition of choline (filled bar) resulted in peroxide formation which was completely abolished in the presence of 2000 U/ml catalase (gray bar). Inclusion of 5 ug/ml non-polar blueberry fraction (NP_{BB}) in the choline oxidation assay was ineffective in scavenging peroxide formation. Note that the lower concentration of NP_{BB} (5 ug/ml) completely abolished ROS formation in SH-SY5Y cells exposed to TNF-α or PMA. In contrast, 75 ug/ml of polar blueberry fraction (PO_{BB}, $^{**}p<0.05$ significantly different than 75 ug/ml NP_{BB}) exhibited significant peroxide scavenging capacity, which was not observed at a fifteen-fold lower concentration (5 ug/ml). All data represent the mean of at least four independent experiments ± standard deviations, and statistical significance was determined at $^{*}p<0.05$ (ANOVA and Tukey's *post hoc* analysis).

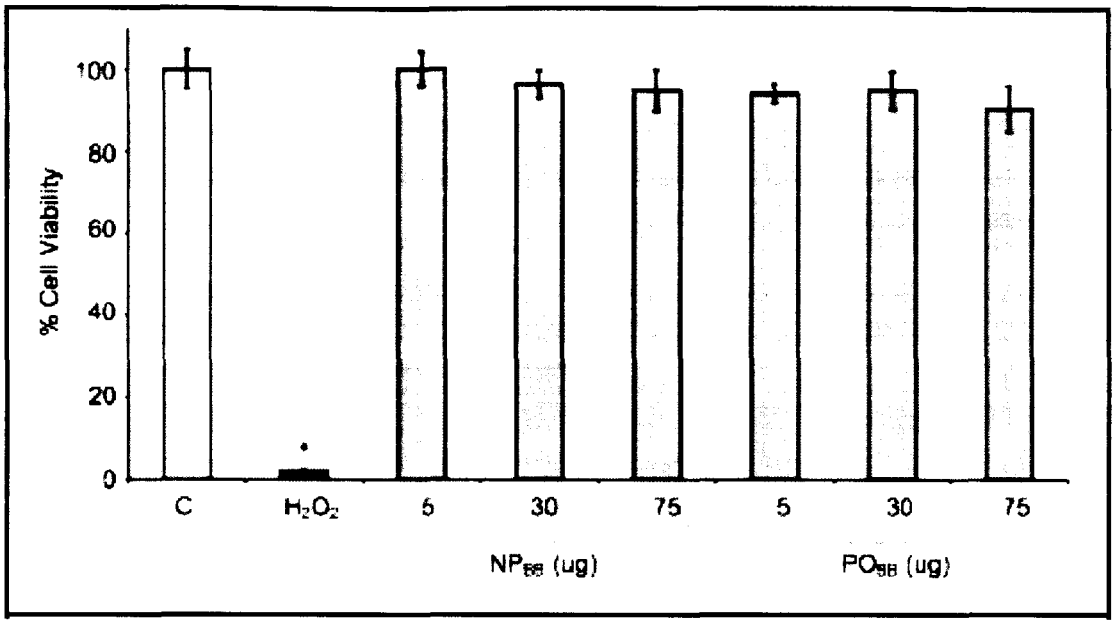
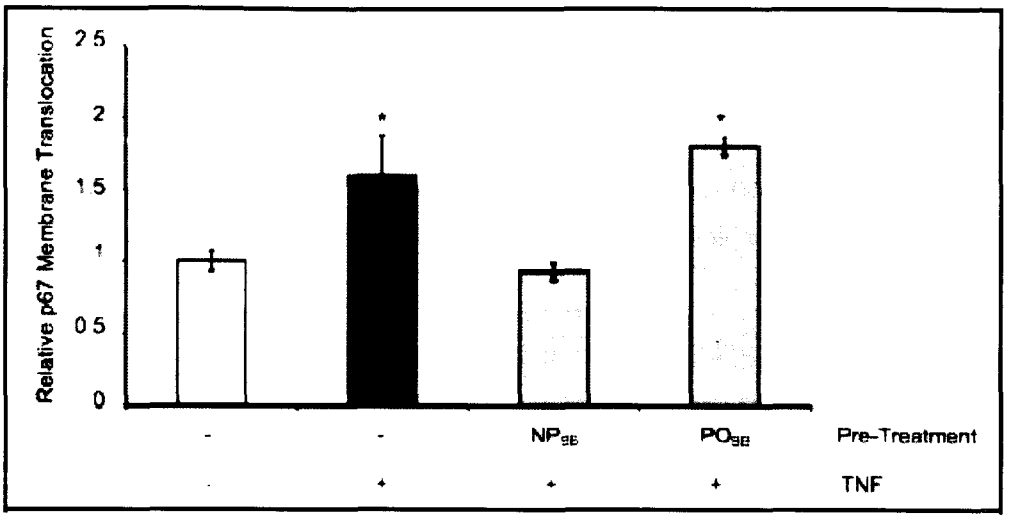


Figure B-4 Non-polar and polar blueberry fractions are not cytotoxic. Serum free cultures of SH-SY5Y cells were supplemented with 5, 30, and 75 ug/ml of non-polar blueberry fraction (NP_{BB}) or polar blueberry fraction (PO_{BB}) for 48 h. Cell viability was measured using a MTT assay and all values were normalized to control (C, open bar). Neither NP_{BB} or PO_{BB} blueberry fractions compromise cell viability at concentrations shown to negate ROS formation in SH-SY5Y cells exposed to TNF-α (200 ng/ml) or PMA (400 ng/ml), compared to our positive control (H_2O, 48 h, filled bar). Data represent the mean of eight independent experiments ± standard deviations, and statistical significance was determined at *$p<0.05$ (ANOVA and Tukey's *post hoc* analysis).

A



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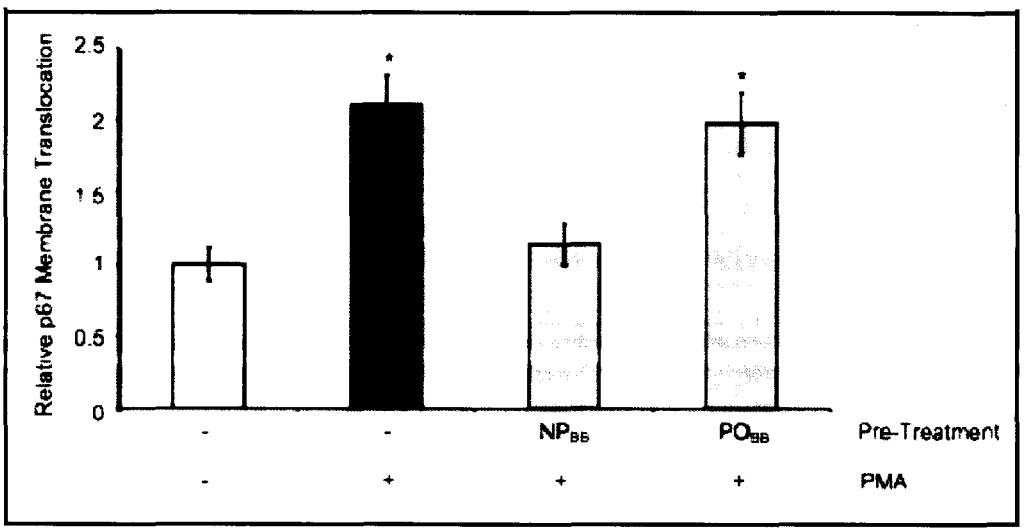
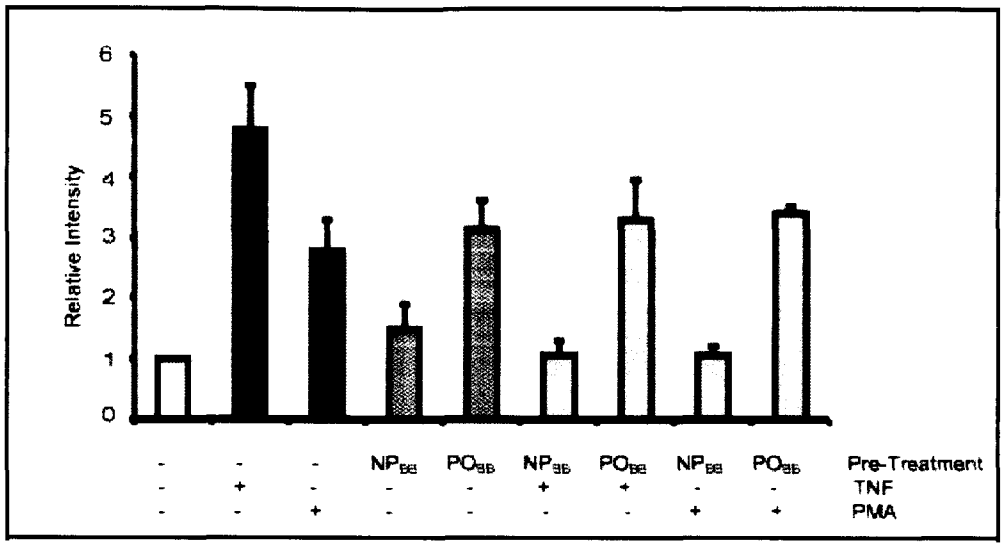


Figure B-5 Non-polar blueberry fraction abolishes $p67^{phox}$ accumulation in plasma membrane. Serum free cultures of SH-SY5Y were incubated 1 h with non-polar (NP_{BB}) and polar (PO_{BB}) blueberry fractions (5 ug/ml each, 1 h) prior to TNF-α (200 ng/ml) or PMA (400 ng/ml) exposure (30 mins). Cells were lysed and fractionated into a cytosolic and membrane fraction. Total membrane protein was absorbed on a 96 well pate (20 ug/ml, overnight). After blocking (5% BSA, 1 h) wells were incubated with rabbit anti-$p67^{phox}$ antibody (1:1000 in 1X TBST, 4° C, overnight) and followed by incubation with goat-anti rabbit secondary antibody conjugated to HRP (1:2000 in 1X TBST, 45 mins,

room temperature). After addition of TMB (100 ul/well) max absorbance was measured at 620 nm, as an indicator of $p67^{phox}$ in the plasma membrane fraction. (A) TNF-α exposure of SH-SY5Y cells caused a significant increase in $p67^{phox}$ in plasma membrane (filled bar) which was negated by the presence of non-polar blueberry fraction (NP_{BB}) compared to control (open bar) whereas polar blueberry fraction (PO_{BB}) was ineffective. (B) PMA exposure of SH-SY5Y cells also resulted in a significant increase in relative absorbance (filled bar), and was negated by 5 ug/ml of non-polar blueberry fraction (NP_{BB}) compared to control (open bar). Polar blueberry fraction (PO_{BB}) did not negate PMA mediated $p67^{phox}$ membrane translocation, compared to control (open bar). All values were normalized to control, data represent the mean of at least four independent experiments ± standard deviations, and statistical significance was determined at $*p<0.05$ (ANOVA and Tukey's *post hoc* analysis).

A



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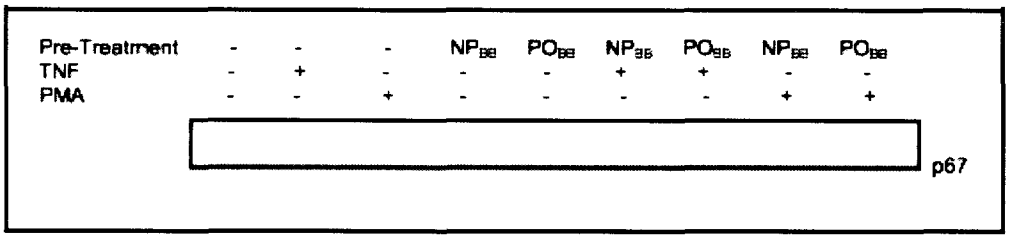

Figure B-6 Non-polar blueberry fraction inhibits translocation of $p67^{phox}$ into plasma membranes. Serum free cultures of SH-SY5Y cells were incubated with non-polar (NP_{BB}) and polar (PO_{BB}) blueberry fractions (5 ug/ml each) 1 h prior to insult with 200 ng/ml TNF-α or 400 ng/ml PMA for 30 mins. Cells were lysed and fractionated into a cytosolic and membrane fraction. Equal amounts of total plasma membrane protein were subjected to SDS gel electrophoresis followed by western blotting and detection of immunoreactivity against $p67^{phox}$ (colorimetric detection). Band intensities were quantified by densitometry (ImageJ64) and all values were normalized to control. TNF-α and PMA (filled bars, respectively) induced $p67^{phox}$ translocation, which was abolished in the presence of non-polar blueberry fraction (NP_{BB}) compared to control whereas polar blueberry fraction (PO_{BB}) was ineffective. Note that incubation of SH-SY5Y cells with NP_{BB} or PO_{BB} alone and in the absence of any insult induced a significant $p67^{phox}$

translocation. All data represent the mean of at least four independent experiments ± standard error of the mean (SEM), and statistical significance was determined at *$p<0.05$ (ANOVA and Tukey's *post hoc* analysis).

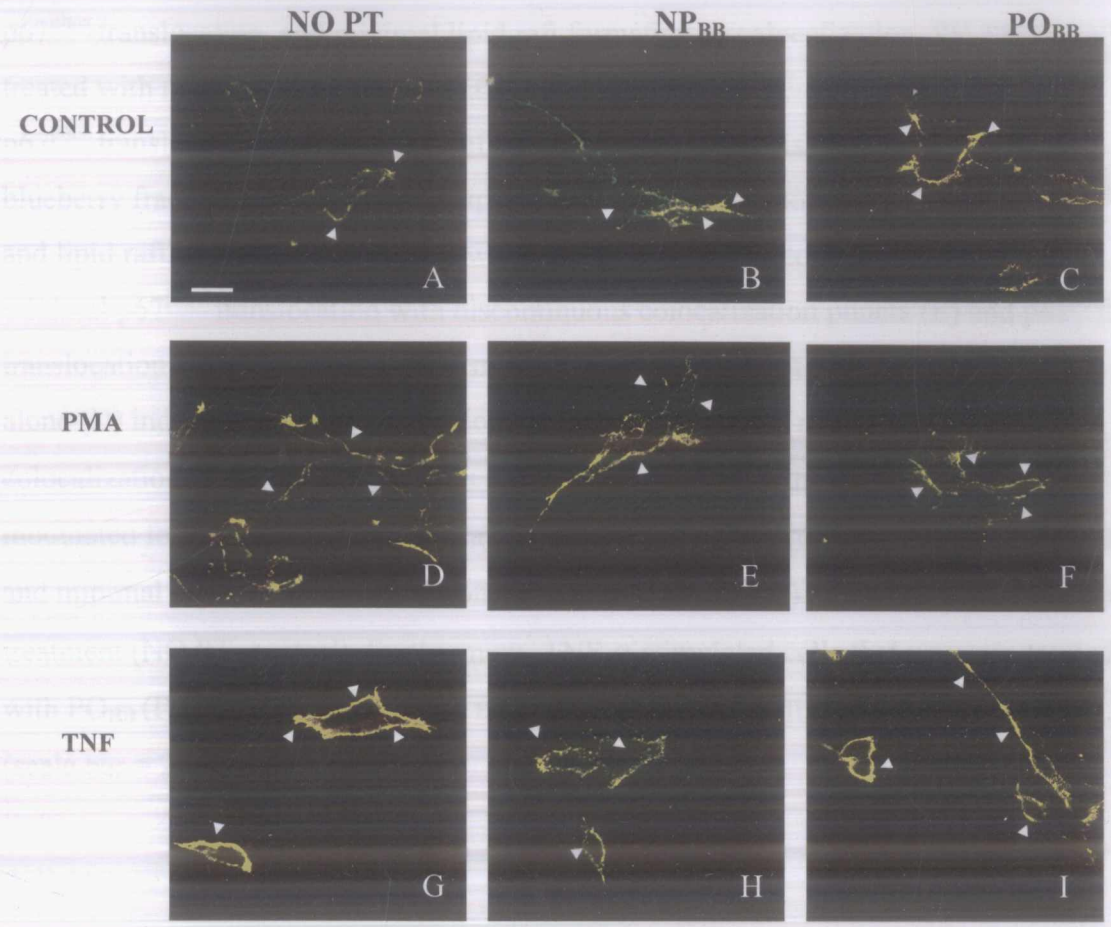

Figure B-7 Non-polar blueberry fraction inhibits the association of p67^{phox} to the plasma membrane by modulating lipid raft platforms. SH-SY5Y cells were plated and grown on poly-lysine coated glass cover slips until 50% confluent. Cells were serum-starved overnight and then incubated with non-polar (NP_{BB}) and polar (PO_{BB}) blueberry fractions (5 ug/ml each) 1 h prior to insult (200 ng/ml TNF-α or 400 ng/ml PMA, respectively) for 30 mins. Cells were then labeled using an Alexa Fluor 555 lipid raft labeling kit, fixed, and permeabalized. Cultures were incubated with anti-p67^{phox} primary antibody and then mounted with PVA-DABCO. Images, acquired using confocal microscopy, show lipid raft platforms (red), p67^{phox} (green) and colocalization of lipid rafts and p67^{phox} (yellow). As expected, TNF-α induced continuous lipid raft formation, p67^{phox} translocation to the plasma membrane, and colocalization of p67^{phox} and lipid rafts

(G) compared to NO PT (no pre-treatment, control, A). PMA treated cells (D) show $p67^{phox}$ translocation, and minimal lipid raft formation or colocalization. SH-SY5Y cells treated with non-polar blueberry fraction (NP_{BB}) showed minimal raft formation and $p67^{phox}$ translocation in the absence of insult (B) where as cells treated with polar blueberry fraction (PO_{BB}) did form lipid raft platforms and show colocalization of $p67^{phox}$ and lipid rafts (C). Cells pre-treated with NP_{BB} or PO_{BB} prior to PMA insult show minimal $p67^{phox}$ translocation with discontinuous colocalization puncta (E) and $p67^{phox}$ translocation with some lipid raft formation and colocalization (F), respectively. PMA alone (D) induced $p67^{phox}$ translocation and shows some lipid raft formation and colocalization. NP_{BB} pre-treated cells that were stimulated with TNF-α (H) had modulated formations of lipid raft platforms marked by discontinuous colocalized puncta and minimal $p67^{phox}$ translocation compared to the TNF-α (G) treated cells without a pre-treatment (NO PT, control). Furthermore, TNF-α stimulated cells that were pre-treated with PO_{BB} (I) did not show the same modulation effects as cells pre-treated with NP_{BB}. (scale bar = 20 μm).

Appendix C

Ursolic Acid Isolated From Lipophilic Blueberry Fraction Inhibits NADPH Oxidase by Lipid Raft Modulation *

C.1 Abstract

The role of NADPH Oxidase (NOX) in neuroinflammation is vast and contributes to a multitude of acute and chronic central nervous system (CNS) pathologies (Harraz et al. 2008). The functional assembly of the NOX enzyme in neuronal cells is marked by the translocation of cytosolic factors ($p67^{phox}$, $p47^{phox}$, $p40^{phox}$) to their membrane bound counterparts ($gp91^{phox}$, $p22^{phox}$) and ultimately to the generation of superoxide anion. Membrane bound NOX subunits have been identified in cholesterol rich domains of neuronal cell plasma membranes, otherwise known as lipid raft (LR) platforms (Vilhardt et al. 2004). LR platforms provide scaffolding properties for redox signaling that promote translocation of specific NOX proteins and orchestrate the functional assembly of NOX (Klopfenstein et al. 2002). It is documented that lipophilic Alaskan blueberry fractions decrease NOX activity by inhibiting the translocation of $p67^{phox}$ to the plasma membrane. In this study we explore the effects of Ursolic acid (Rizzo 2006) isolated from a highly lipophilic fraction of wild Alaskan Blueberries on $p67^{phox}$ translocation. Our findings suggest that the ability of UA to inhibit $p67^{phox}$ translocation is intimately linked to modulation of LR platforms in neuroblastoma cells exposed to tumor necrosis factor alpha (TNF-α). This investigation validates NOX as a molecular target of neuroinflammation and validates deregulation of NOX by the effects of UA on the plasma membrane of neuronal cells.

* Manuscript in preparation for submission. Gustafson, SJ, Hogan, MB, McGill, CM, Kuhn TB.

C.2 Introduction

NADPH Oxidase (NOX) is a multimeric enzyme that requires the assemblage of at least two membrane-bound subunits ($gp91^{phox}$ and $p22^{phox}$), and three cytosolic subunits ($p67^{phox}$, $p47^{phox}$, and $p40^{phox}$). The translocation of the cytosolic subunits to their membrane bound counterparts is phosphorylation dependent and relies on protein-lipid interactions of specific membrane targeting domains (Groemping et al. 2005; Ueyama et al. 2007). Once assembled, NOX generates superoxide, a specific type of reactive oxygen species (ROS) that occurs in an acute immune response to mediators such as eicosanoids and inflammatory cytokines (Kim et al. 2008). The inflammatory cytokine known as tumor necrosis factor alpha (TNF-α) elicits cellular signaling that leads to oxidative stress associated with various neurodegenerative conditions (Block et al. 2007).

Eukaryotic cell membranes are comprised of several active domains characterized by their distinct physical and biological properties. Lipid raft (LR) platform domains are cholesterol and sphingolipid rich regions (Rizzo 2006) known to provide scaffolding properties and contribute to redox signaling events within the plasma membrane (Vilhardt et al. 2004); (Klopfenstein et al. 2002; Klopfenstein et al. 2002). The recruitment and assembly of cytosolic NOX proteins are intimately linked to membrane bound NOX subunits that co-localized within LR domains (Eum et al. 2009; Li et al. 2009).

The integration of molecular targets, such as NOX, within LR domains makes them an area of interests in the quest to reduce oxidative stress associated with neurodegeneration. Compounds such as ursolic acid that are lipophilic in nature and have similar molecular characteristics to cholesterol may have the ability to replace or displace cholesterol in LR domains thus change the composition of the plasma membrane and intervene with the function of molecular targets that reside in this cellular compartment. This study aims to examine the effects of ursolic acid, isolated from lipophilic fractions of wild Alaska bog blueberries, on NOX function through modulation of LR platforms in the plasma membrane of neuroblastoma cells exposed to TNF-α.

C.3 Experimental Procedures

C.3.1 Reagents

Recombinant human tumor necrosis factor alpha (TNF-α) was purchased from Millipore (Temecula, CA). DMEM and Penicillin/Streptomycin solution were obtained from Mediatech (Herndon, VA). GlutaMAX-1, trypsin/EDTA solution, TMB, NuPAGE running and transfer buffers, and Alexa Fluor 555 Lipid Raft labeling kit were from Invitrogen (Carlsbad, CA). Fetal bovine serum was received from Atlanta Biologicals (Atlanta, GA). BCA protein assay kit and 1-step NBT/BCIP were from Pierce (Rockford, IL). All other reagents were purchased from Sigma (St. Louis, MO).

C.3.2 Cell Culture

Human SH-SY5Y neuroblastoma cells were grown in 100 mm dishes (falcon) in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 U/ml streptomycin (humidified atmosphere, 5% CO_2, 37°C). For amplification, SH-SY5Y cells were treated with trypsin (0.5 mg/ml)/EDTA (0.2mg/ml) for 5 min, rinsed, and immediately resuspended in growth medium onto poly-lysine coated glass cover slips in 35 mm dishes (confocal microscopy) or into tissue culture treated plates. Cells were grown for 24 h to 40% (confocal microscopy) or 80% confluency and were then serum starved overnight prior to experimentation.

C.3.3 Cell Viability

Cultures of SH-SY5Y were serum deprived overnight followed by treatments of H_2O_2, and 5, 250, and 500 ug/ml ursolic acid for 48 h. Potential toxic effect on cell viability were monitored using a MTT assay according to manufactures instructions (Millipore) and formazan generation was measured using a Beckman Coulter Multimode DTX 880 microplate reader.

C.3.4 Enzyme-linked Immunosorbent Assay (ELISA) for $p67^{phox}$

Cultures of SH-SY5Y cells were grown in 100 mm dishes to confluency and serum deprived overnight. Cells were subjected to 1 h pre-treatments of isolated and pure Ursolic acid (5 ug/ml) prior to exposure of TNF-α (200 ng/ml) for 30 mins. Cells were harvested and a cellular fractionation was performed to obtain cytosolic and plasma membrane fractions. Protein quantification of the membrane fraction was determined using a BCA assay (Pierce) and 20 ug of protein was plated to a 96 well high protein absorbent plate (Falcom) overnight. Wells were blocked with 5% BSA in 1X TBST (1 h) and then incubated with $p67^{phox}$ primary antibody (3 ug/ml, 4° C, overnight). Next, cells were washed with 1X TBST (3 times, 5 mins each), and incubated with corresponding secondary antibody (1:2000, 45 mins, room temp) conjugated to HRP. Well were rinsed and addition of TMB (100 ul/well, 10 mins) allowed for colorimetric analysis using a Beckman Coulter Multimode DTX 880 microplate reader to measure the absorbance at 620 nm.

C.3.5 Cellular Fractionations

Cells were grown to confluency, treated, harvested and sonicated in a sucrose buffer (20 mM Tris-HCL, 2 mM EDTA, 0.5 EGTA, 2 mM AEBSF, 25 ug/ml Leupeptin, 0.33 M Sucrose, pH 8.0). Suspensions were centrifuged at 14000 rpm for 15 mins (Labnet spectrafuge 16M) and the supernatant was removed (cytosolic fraction). The pellet was then resuspended and sonicated in a buffer containing 20 mM Tris-HCL, 2 mM EDTA, 0.5 mM EGTA, and 2 mM AEBSF, pH 8.0. Cells were centrifuged again for 15 mins and the supernatant was collected (plasma membrane). A micro BCA protein analysis (pierce) was used according to the manufacturer's instructions to determine the protein concentration for each sample.

C.3.6 Detection of Lipid Rafts Labeled with Alexa Fluor 555

Cells were grown to confluency, treated, labeled with Alexa Flour 555 (according to the manufacturer's instructions), harvested in a sucrose buffer and fractionated into

cytosolic and membrane fractions as described above. The membrane fraction was then subjected to A/G Agarose beads (100 ul, 30 mins, on ice), centrifuged (200 xg_{max}, 2 mins) and the supernatant was discarded. The pellet was resuspended in 1% Triton buffer, centrifuged, and the supernatant was removed (detergent soluble membrane, DSM). The pellet was resuspended in 1X PBS as the detergent resistant membrane (DRM) fraction and equal amounts of DRM protein for each treatment were plated to a black 96 well dish (falcon). Fluorescence detection of Alexa Fluor 555-labeled lipid rafts were measured using a Beckman Coulter Multimode DTX 880 microplate reader.

C.3.7 SDS Polyacrylamide Gel Electrophoresis

Cultures of SH-SY5Y cells were treated, harvested in sucrose buffer, and fractionated into cytosolic and membrane fractions. Total protein concentrations for membrane fractions were determined using a BCA protein assay kit. Equal amounts of membrane protein (2 μg) were loaded onto 15% polyacrylamide gels, and subjected to electrophoresis under denaturing conditions.

C.3.8 Western Blotting

Proteins were transferred from Polyacrylamide gels onto nitrocellulose membranes (2.5 h, 125 volts, 50 watts, 250 mA) and membranes were blocked with 5% w/v BSA in 1X TBST (50 mM TRIS HCl, pH 7.4, 150 mM NaCl, and 0.1% v/v Tween 20). Membranes were probed with primary antibodies (1:500) in TBST overnight, washed with TBST (3 times, 5 minutes each), and incubated with alkaline phosphatase-conjugated secondary antibodies (1:5000 in TBST) for 45 minutes. Immunoreactivity was detected by colorimetric detection using NBT/BCIP (Pierce) according to the manufacture's direction. Band intensities were quantified using ImageJ-64.

C.3.9 Confocal Microscopy

Human SH-SY5Y neuroblastoma cells were grown on poly-lysine coated glass cover slips (0.13 mm thick German glass) in medium for 24 h. Cells were serum-starved

overnight and incubated 1 h UA (5 ug/ml) prior to acute addition of TNF-α (200 ng/ml). Cultures were labeled with Alexa fluor Lipid Raft Labeling kit 555 (Invitrogen) according to manufactures instructions. Cultures were rinsed with 1X TBS and cover slips were mounted with PVA-DABCO. After drying overnight at room temperature, slides were stored at 4°C until image analysis. Images were acquired (63x, oil, Plan Fluor) with a Zeiss confocal microscope LSM 510 equipped with a He/Ne laser and an Argon laser using 554 excitation and 570 emission filters. Zeiss LSM Software was used for image acquisition and analysis. For each treatment condition, random fields of view were analyzed and 3 cells from two independent sets of experiments ($n = 6$) were examined for the presence of lipid raft platforms (red).

C.3.10 Statistical Analysis

Analysis of variance (ANOVA) was used to determine significance among treatments ($p < 0.05$) followed by Tukey's Post hoc analyses performed using Statistical Analysis System (SAS). All values are expressed as mean values $\pm$ standard deviations or standard error of the mean.

C.4 Results

The exposure of neuronal cells to TNF-α elicits cellular signaling that guides the functional assembly of NADPH Oxidase (NOX) and subsequent formation of superoxide. In previous studies we demonstrated that blueberry fractions with a non-polar nature inhibited NOX activity by preventing TNF-α-mediated translocation of $p67^{phox}$ to the plasma membrane in SH-SY5Y human neuroblastoma cells. Here, we tested whether ursolic acid (Rizzo 2006) has the capacity to inhibit $p67^{phox}$ translocation to the plasma membrane of neuronal cells exposed TNF-α. Quantification of $p67^{phox}$ translocation was determined by ELISA for $p67^{phox}$ in the plasma membrane of SH-SY5Y neuroblastoma cells. As shown in Figure 1, SH-SY5Y cells subjected to a 30 min exposure of 200 ng/ml TNF-α revealed a dramatic increase in $p67^{phox}$ translocation (1.35±0.03, n=4, *$p<0.05$) compared to control cultures (1.00±0.01, n=4). In contrast, the presence of UA isolated

from wild Alaska bog blueberries (IC UA) and commercially available UA (pure UA) at 5 ug/ml both negated TNF-α-mediated $p67^{phox}$ translocation to the plasma membrane (1.13±0.04, n=4, $p<0.05$ and 1.12±0.03, $p<0.05$, respectively). In addition, we reveal that UA isolated from wild Alaska bog blueberries has the capacity to inhibit $p67^{phox}$ translocation in detergent resistant membrane fractions (Fig. 3B) of plasma membranes isolated from SH-SY5Y cells exposed to TNF-α.

We next examined the effects of ursolic acid, a compound that is structurally similar to cholesterol, on LR platforms in detergent resistant membranes (DRM) of neuronal cells exposed to TNF-α (Fig. 2). As expected, SH-SY5Y cells exposed to 10 uM of MBCD for 10 mins alone or prior to 30 mins of 200 ng/ml TNF exposure resulted in a significant decrease in LR formation (0.54±0.36, n=6, $p<0.05$ and 0.87±0.15, n=6, $p<0.05$, respectively) compared to cells exposed TNF (1.98±0.32, n=6, $p<0.05$). Cells pre-treated with a 10 uM of a sphingomyelinase inhibitor, GW4869 (GW), or with 5 ug/ml of UA for 1 hour prior to TNF-α exposure both show decreased presence of LR platforms in DRM (0.60±0.07, n=6, $p<0.05$ and 1.07±0.21, n=6, $p<0.05$, respectively) when compared to control cells (1.00±0.08, n=6, $p<0.05$). Notably, cells exposed to UA in the absence of insult did not increase LR presence in DRM (0.84±0.12, n=6, $p<0.05$) and exposure of SH-SY5Y cells to MBCD post-UA treatment did not significantly impact LR formations (1.28±0.22, n=6, $p<0.05$) when compared to control cells.

Ganglioside-1 (GM1) and flotillin are biomarkers specific to LR domains of the plasma membrane. As shown in Figure 3, we examined the effects of UA on GM1 (Fig. 3C) and Flotillin (Fig. 3D) in the plasma membrane of neuronal cells exposed to TNF-α. As expected TNF-α exposure (TNF) increased both GM1 and Flotillin in the membrane of SH-SY5Y cells when compared to cells that were not treated (Kellogg et al. 2009) where as MBCD treatments (MBCD and TNF+MBCD) decreased the presence of both GM1 and flotillin (Fig. 3C, 3D). For the first time, we reveal that ursolic acid (Rizzo 2006) decreases TNF-α-mediated GM1 and flotillin in the plasma membranes of SH-SY5Y cells exposed to TNF-α, which suggests a decrease in LR formation. In addition

we validated a decrease in TNF-α-mediated $p67^{phox}$ translocation to the plasma membrane of SH-SY5Y cells pre-treated with UA (Fig. 3B). Note that western blot analysis of gp91 (Fig. 3A) served as an experimental control.

The viability of human SH-SY5Y neuroblastoma cells upon prolonged exposure to UA was determined to verify the specificity of UA on decreased $p67^{phox}$ translocation and LR detection. Figure 5 demonstrates that UA (5 ug/ml) does not compromise cell viability (0.77±0.21, n=8, $p<0.05$) over a 48 h time period compared to control (1.00±0.09, n=8, $p<0.05$) and even at concentrations up to 100 fold (500 ug/ml) those shown to decrease $p67^{phox}$ translocation and LR formations (75 ug/ml: 0.82±0.18, n=8, $p<0.05$; 250 ug/ml: 0.85±0.06, n=8, $p<0.05$; and 500 ug/ml: 0.80±0.14, n=8, $p<0.05$). As expected H2O2 (48 h) caused a drastic reduction in cell viability (0.13±0.12, n=8, $p<0.05$, positive control). This result demonstrates that UA did not compromise SH-SY5Y cell viability at concentrations that inhibited $p67^{phox}$ translocation and decreased LR detection.

Lastly, we show the ability of UA to modulate LR platforms in neuroblastoma cells exposed to TNFα using confocal microscopy (Fig. 5). Control cells (No Treatment) show minimal LR presence compared to cells exposed to 200 ng/ml TNF-α for 1 hr. SH-SY5Y cells subjected to MBCD show a significant decrease in LR presence while cells treated with 5 ug/ml UA prior to insult with TNF appear to have modulated LR platforms.

C.5 Discussion

High levels of oxidative stress related to neuroinflammation have been linked to the release of proinflammatory cytokines such as tumor necrosis factor alpha (TNF-α), by macrophages of the CNS (Tezel 2008). TNF-α is known to activate magnesium-dependent neutral sphingomyelinase (Mg^{2+}-nSMase) and subsequently liberate ceramide from sphingomyelin in the plasma membrane (Wheeler et al. 2009). In due course, secretion of TNFα orchestrates the progression of neuronal degeneration (Li et al. 2009) through ceramide-dependent cellular stress responses including excessive ROS

formation, functional assembly of NADPH Oxidase (NOX), and increased oxidative stress – all of which lead to neurodegeneration. The TNF-α-dependent recruitment of TNF-α-receptor-1 (TNF-R1) into lipid raft (LR) platforms validates the significant role of TNF-α in the formation of LR platforms of plasma membranes (Doan et al. 2004). LR platforms are cholesterol and sphingolipid rich regions of the cellular plasma membrane that provide scaffolding properties for cellular signaling and for the assembly and function of membrane proteins such as NOX.

Our findings reveal for the first time, that ursolic acid (Rizzo 2006) intervenes with the functional assembly NOX in a neuronal model of inflammation. Here, we demonstrate that two types of ursolic acid (Rizzo 2006), one isolated from wild Alaska bog blueberries and the other commercially available, both inhibit the translocation of $p67^{pohx}$ (Fig. 1; Fig. 3B). Next, we isolated detergent resistant membranes (DRM) from whole cell lysates of SH-SY5Y cells to examine the effects of UA on LR platforms. In this experiment (Fig. 2), ganglioside-1 (GM1) was tagged with Alexa Fluor 555 labeled cholera toxin (CTx), crosslinked, isolated, and quantified by fluorescence detection. The structural integrity of LR platforms is compromised when exposed to cholesterol-sequestering agents such as Methyl-β Cyclodextrin (MBCD) (Yang et al. 2007). The prevalence of cholesterol in LR platforms permitted the use of MBCD as a suitable control for this experiment. Disruption of LR platforms by cholesterol depletion prevents TNF-α-dependent recruitment of TNF-R1 to LR platforms (Doan et al. 2004) and thus decreases the presence of GM1 in DRM (Fig. 2). Perturbation of LR composition may also alter the function of proteins, such as NOX, that are dependent on LR domains as signaling platforms.

The structural framework that LR domains provide for a variety cellular and molecular signaling creates an ideal environment for the NOX membrane subunits to reside. In fact, the co-localization of $gp91^{phox}$ and $p22^{phox}$ within LR platforms may be crucial for subunit interaction and for the translocation of NOX cytosolic subunits to their membrane bound counterparts. Here, we quantitatively measured $gp91^{phox}$ as a control for plasma membrane protein and show again that UA prevents the translocation of $p67^{phox}$ to

the plasma membrane of SH-SY5Y cells exposed to TNF-α (Fig. 3B). The ganglioside GM1 is abundant in plasma membranes of nerve cells and is known to preferentially partition into LR platforms hence the use of GM1 as a popular LR biomarker. Flotillin, also prominently found in LR platforms of the plasma membrane, serves as an additional biomarker for LR formation. Using western blot analysis, we quantitatively measured the presence GM1 and flotillin (Fig. 3C, 3D) in SH-SY5Y cellular membranes treated with UA prior to TNF-α exposure. The decrease of GM1 and flotillin in the plasma membrane of SH-SY5Y cells subjected to MBCD demonstrates the ability of MBCD to negate LR platforms. In addition, we show that UA treatments also reduce LR platforms in SH-SY5Y cells exposed to TNF-α and that UA is not cytotoxic in this neuronal model of inflammation (Fig. 4). Moreover, we demonstrate via cellular imaging that the capacity of UA to inhibit $p67^{phox}$ translocation is interrelated to the modulation of LR platforms in the plasma membranes of SH-SY5Y human neuroblastoma cells exposed to TNF-α (Fig. 5). In conclusion, we demonstrate UA intervention of LR platforms in plasma membrane using DRM isolation, western blot analysis, and confocal microscopy.

Natural compounds isolated from a variety of plants have shown exemplary health benefits. For example, plant sterols such as beta-sitosterol (Structure 1) have been shown to lower cholesterol levels in human studies (Cicero et al. 2002). The bioactivity of natural compounds that comprise wild Alaskan blueberries are also well documented and are suggested to ameliorate metabolic disorders such as obesity and diabetes (Kellogg et al. 2009). Compounds such beta-sitosterol, and UA (Structure 2) isolated from wild Alaska bog blueberries, exhibit structural and dynamic similarities to that of cholesterol (Structure 3) and may have the ability to replace or displace cholesterol in the plasma membrane of various cell types. Replacing or displacing cholesterol can compromise the structural integrity of LR platforms and can influence membrane curvature. We speculate that modulation of LR platforms by UA is due to the structural similarities of UA and cholesterol. Altogether, this research identifies neuronal NOX as a specific molecular target for nutrition-based strategies against inflammation associated with acute and chronic pathologies as well as general aging. Identifying the mechanistic

characteristics in which UA modulates LR platforms could provide further insight into the inhibition of NOX-mediated neuronal inflammation.

C.6 Acknowledgements

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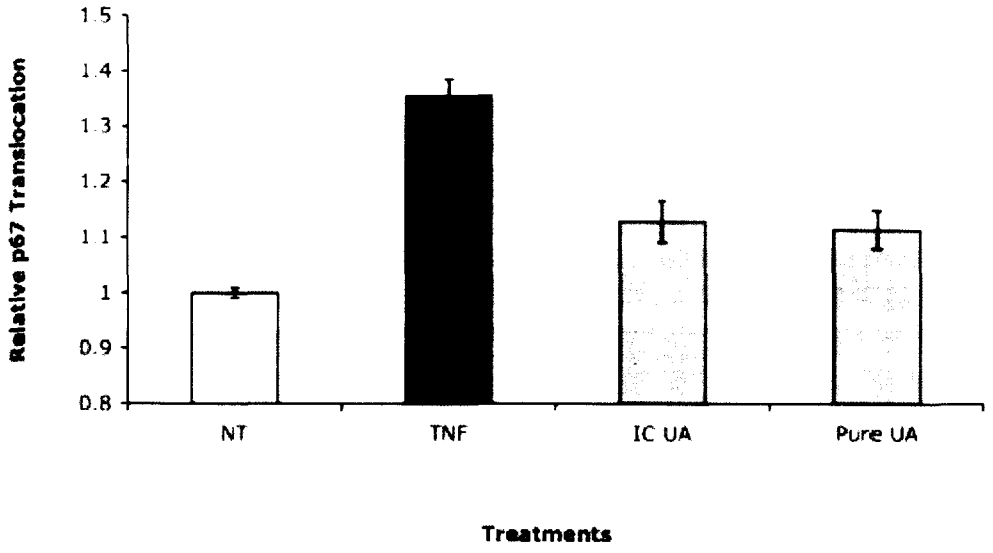

Figure C-1 Ursolic acid abolishes $p67^{phox}$ accumulation in plasma membrane. Serum free culture of SH-SY5Y cells were incubated with Ursolic acid isolated from Alaskan blueberries (IC UA) and pure Ursolic acid (Pure UA) (5 ug/ml) for 1 h prior to insult with 200 ng/ml TNFα for 30 mins. Cells were lysed and fractionated into a cytosolic and membrane fraction. Total membrane protein was absorbed on a 96 well plate (20 ug/ml, overnight). After blocking (5% BSA, 1 h) wells were incubated with rabbit anti-$p67^{phox}$ antibody (1:1000 in 1X TBST, 4° C, overnight) and followed by incubation with goat-anti rabbit secondary antibody conjugated to HRP (1:2000 in 1X TBST, 45 mins, room temp). After addition of TMB (100 ul/well) max absorbance was measured at 620 nm, as an indicator or $p67^{pohx}$ in the plasma membrane fraction. TNF-α exposure of SH-SY5Y cells caused a significant increase in $p67^{phox}$ in plasma membrane (filled bar), which was negated by both, isolated and pure Ursolic acid (IC UA, Pure UA, respectively, grey bars) compared to cells not treated (NT, open bar). Error bars represent standard error of the mean of at least four independent experiments and statistical significance was determined at *$p<0.05$ (ANOVA and Tukey's *post hoc* analysis).

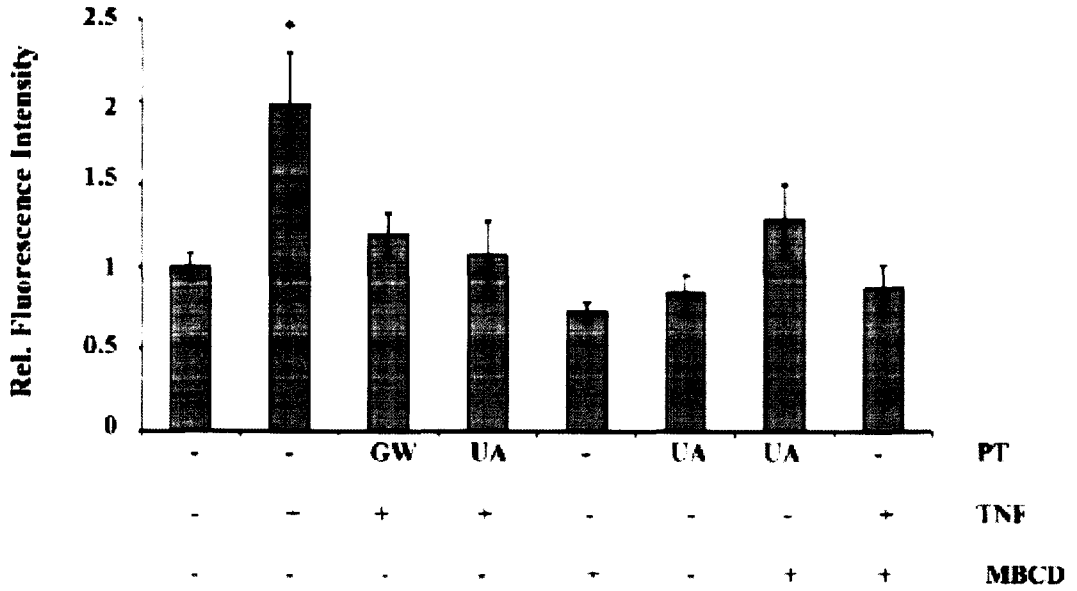

Figure C-2 Ursolic acid abolishes detection of lipid rafts in SH-SY5Y cells exposed to TNFα. SH-SY5Y cells were plated and grown to 90% confluency. Cells were serum starved overnight and then pre-treated (PT) with Ursolic acid (UA) or GW4869 (GW) prior to exposure of TNF-α (200 ng/ml) or MBCD (10 uM). Lipid rafts were labeled with Alexa Fluor 555 – Lipid Raft labeling kit (according to manufactures instructions) and then harvested. Lysates were fractionated into membrane and cytosolic fractions and then the membrane fraction was further separated into a detergent soluble membrane (DSM) fraction and a detergent resistance membrane (DRM) fraction. Equal amounts of protein for each fraction were analyzed for florescence intensity as an indicator for lipid raft detection. As expected, TNF-α exposure increased fluorescence detection of lipid rafts in the DRM compared that of cells not pre-treated (PT) or exposed to TNF-α or MBCD. Cells exposed to MBCD alone-abolished detection of lipid rafts in the DRM as did cells pre-treated with GW and UA prior to TNF-α exposure. Cells pre-treated (PT) with only UA did not have significant effects on lipid raft presence in DRM when compared with control cells nor did treatments of UA+MBCD or TNF-α+M.

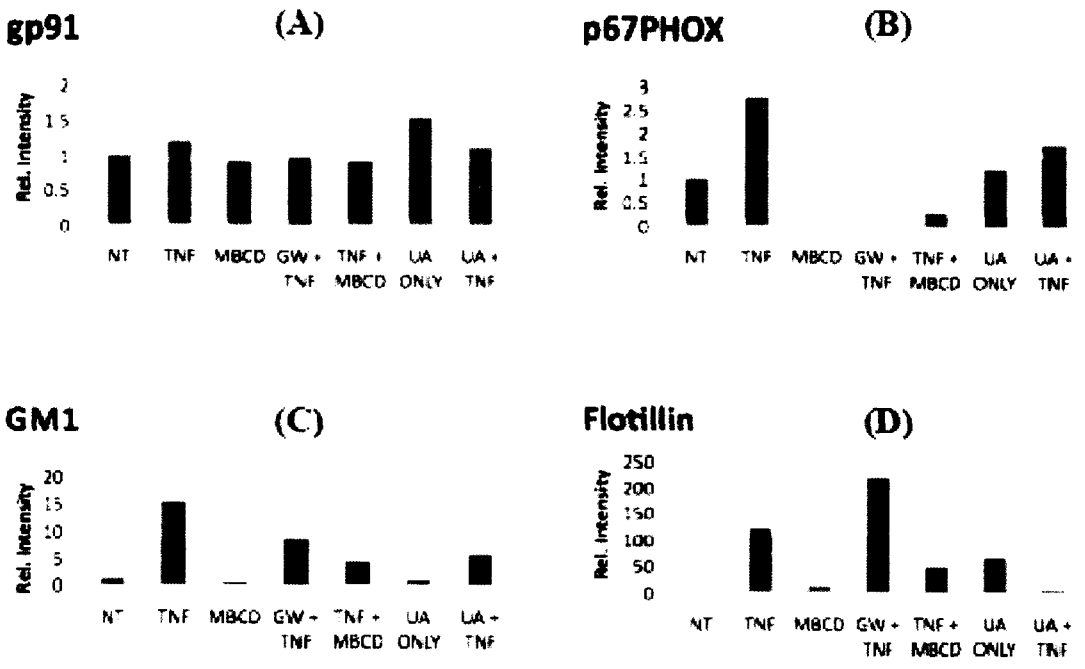

Figure C-3 TNFα increases p67^{phox}, GM1, and Flotillin in the membrane of neuroblastoma cells. Serum free culture of SH-SY5Y cells were incubated with Ursolic acid isolated from Alaskan blueberries (IC UA) and pure Ursolic acid (Pure UA) (5 ug/ml) for 1 h prior to insult with 200 ng/ml TNF-α for 30 mins. Cells were lysed and fractionated into a cytosolic and membrane fraction. Equal amounts of total plasma membrane protein were subjected to SDS gel electrophoresis followed by western blotting and detection of immunoreactivity against gp91, p67, GM1, and flotillin (colorimetric detection). Band intensities were quantified by densitometry (ImageJ64) and all values were normalized to control. (A) All treatments show similar levels of gp91 detected in the plasma membrane. (B) TNF-α (TNF) increased p67^{pohx} presence in the plasma membrane compared to cells not treated (NT). As expected, cells treated with methyl-b-cyclodextrin (MBCD and TNF+MBCD) and with GW4869 (GW+TNF) negated the translocation of p67^{phox} to the plasma membrane. Cells pre-treated with Ursolic acid (UA+TNF) prior to TNF stimulation show decreased p67^{phox} levels

compared to TNF-α alone (TNF). Note that Ursolic acid treatments without stimulation (UA only) did not increase $p67^{phox}$ compared to cells not treated (NT). (C) Cells treated with TNF-α (TNF) increased GM1 in the plasma membrane compared to cells not treated (NT) as well to cells treated with methyl-b-cyclodextrin (MBCD and TNF+MBCD) and those pretreated with GW4869 (GW+TNF). Cells treated with Ursolic acid only (UA only) show minimal GM1 detection while those pre-treated with Ursolic acid prior to TNF-α (UA+TNF) show a decrease in GM1 compared to that of cells treated with TNF-α (TNF). (D) Cells treated with TNFα (TNF) increased Flotillin detection which was abolished by ursolic acid (UA + TNF) compared to cells not treated (NT). Cells treated with Ursolic acid in the absence of insult (UA only) show minimal Flotillin levels whereas pre-incubation of SH-SY5Y cells with GW4869 (GW) prior to TNF-α stimulation induced a significant increase of Flotillin in the plasma membrane.

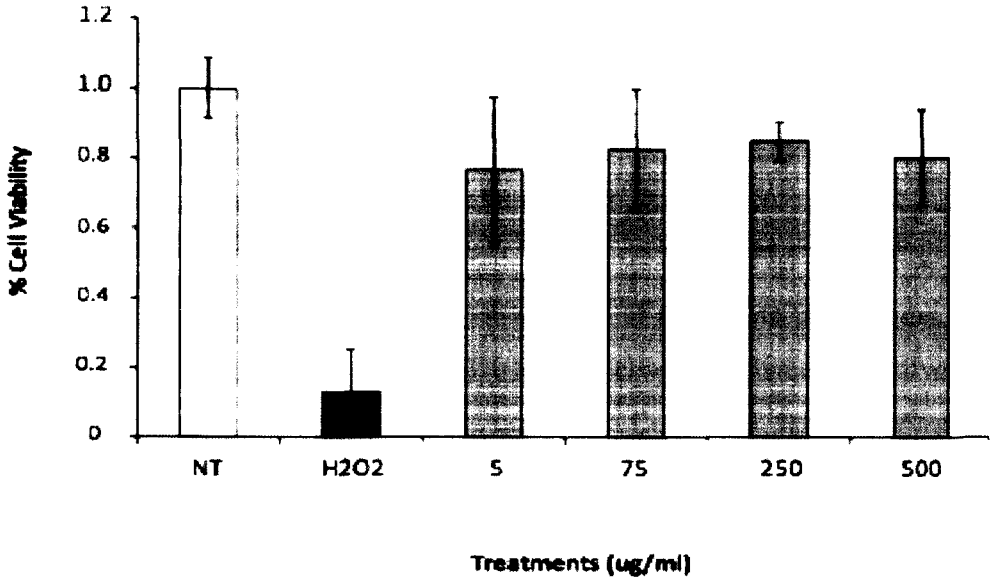

Figure C-4 Pure Ursolic acid is not cytotoxic. Serum free cultures of SH-SY5Y cells were supplemented with Ursolic acid (5, 75, 250, and 500 ug/ml) and maintained for 48 h prior to measuring cell viability (MTT assay). All values were normalized to cells not treated (NT, open bar). Ursolic acid (grey bars) did not compromise cell viability at concentrations (5ug/ml) shown to inhibit $p67^{phox}$ translocation, nor did Ursolic acid compromise cell viability at a 15 (75 ug/ml), 50 (250 ug/ml) or 100 (500 ug/ml) fold excess. As a positive control, H_2O_2 exposure (48 h) of SH-SY5Y cells dramatically reduced cell viability (H_2O_2, filled bar). Error bars represent standard deviations of the mean of at least eight independent experiments and statistical significance was determined at *$p<0.05$ (ANOVA and Tukey's *post hoc* analysis).

Figure C-5 Ursolic acid inhibits the association of $p67^{phox}$ to the plasma membrane by modulating lipid raft platforms. SH-SY5Y cells were plated and grown on poly-lysine coated glass cover slips until 50% confluent. Cells were serum starved overnight and then incubated with Ursolic acid (5 ug/ml) 1 h prior to insult with TNF-α (200 ng/ml) for 30 mins. Cells were then labeled using an Alexa fluor 555-lipid raft labeling kit, fixed, and mounted with PVA-DABCO. Slides were stored at room temperature until imaged using confocal microscopy. Cells with no treatment show minimal lipid raft (red) formation. As expected, methyl-b-cyclodetrin (MBCD) completely abolished any lipid raft platforms present in the plasma membrane of SH-SY5Y cells. Cells treated with TNF-α show significant and continuous lipid raft platforms (red) whereas cells pre-treated with Ursolic acid prior to TNF-α treatment (Ursolic acid + TNF) show modulated lipid raft platforms in the plasma membrane. (Scale bar = 20 um).

Appendix D

Supplementary NMR spectra for β-sitosterol

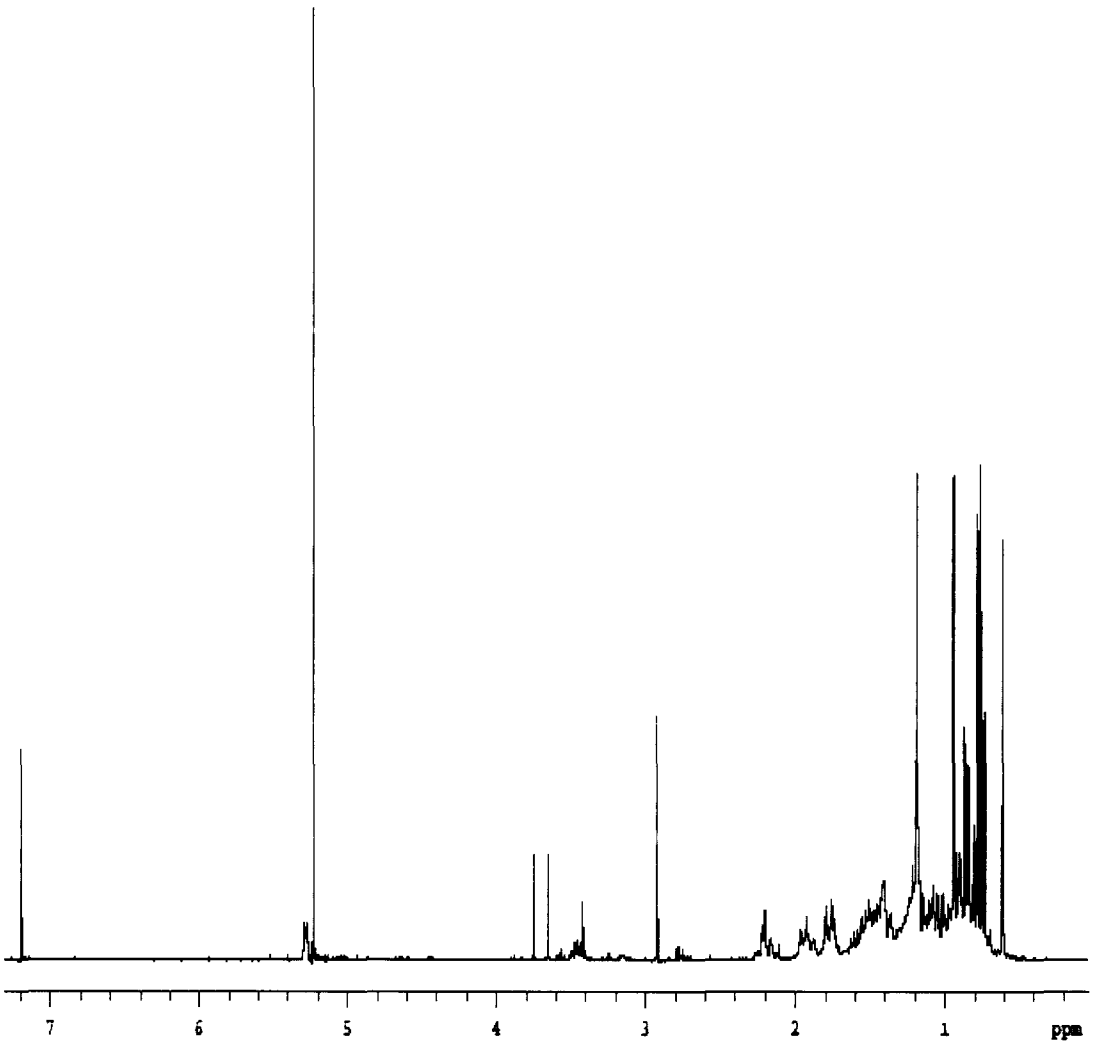

Figure D-1 ^{1}H NMR of β-sitosterol isolated from fraction 6.

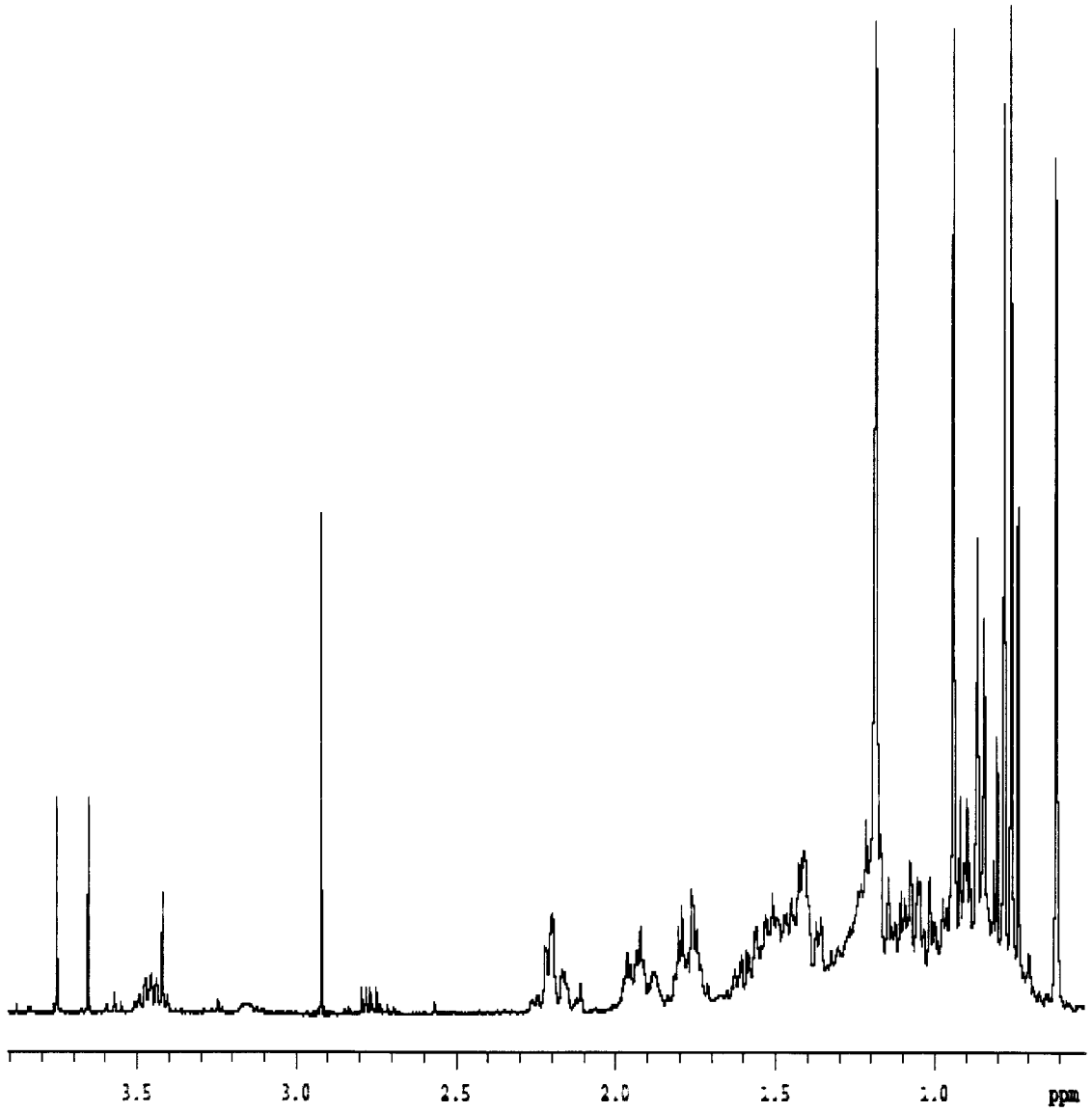

Figure D-2 Upfield region of ^{1}H of β-sitosterol isolated from fraction 6.

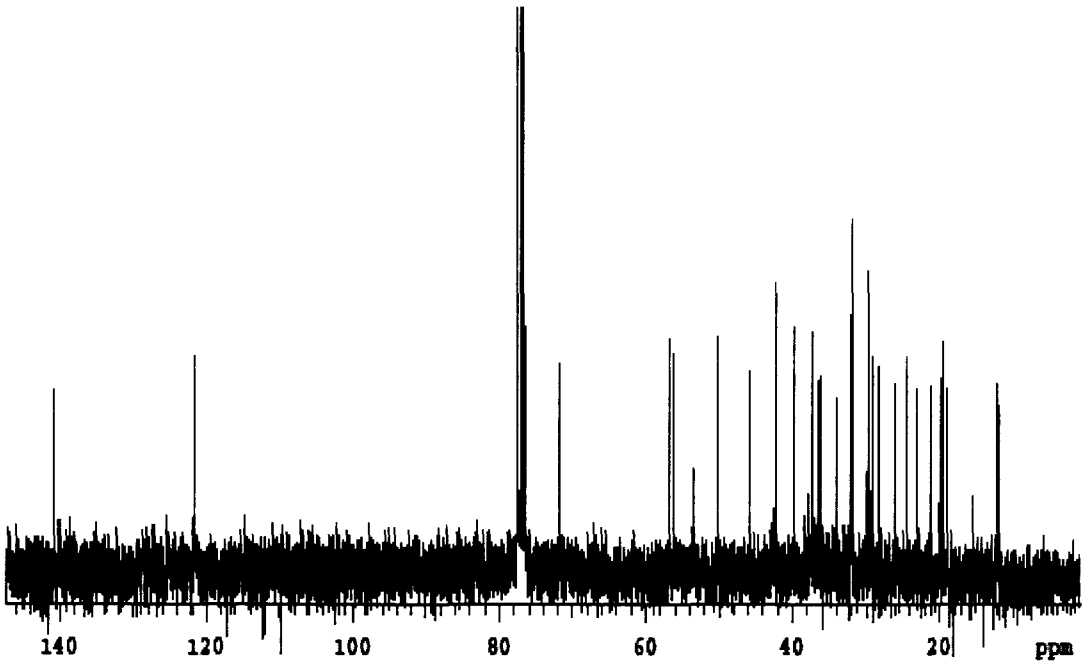

Figure D-3 Full ^{13}C NMR of β-sitosterol isolated from fraction 6.

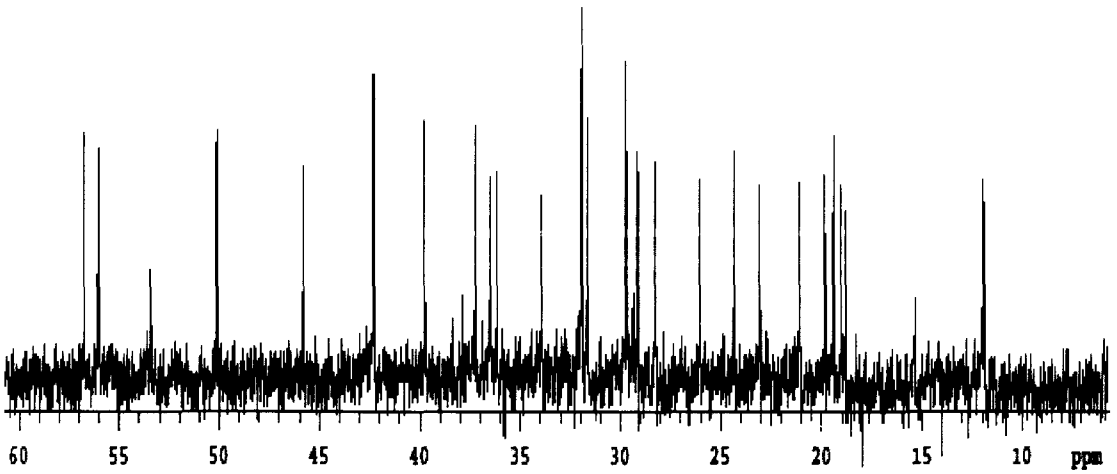

Figure D-4 Upfield ^{13}C NMR of β-sitosterol isolated from fraction 6.

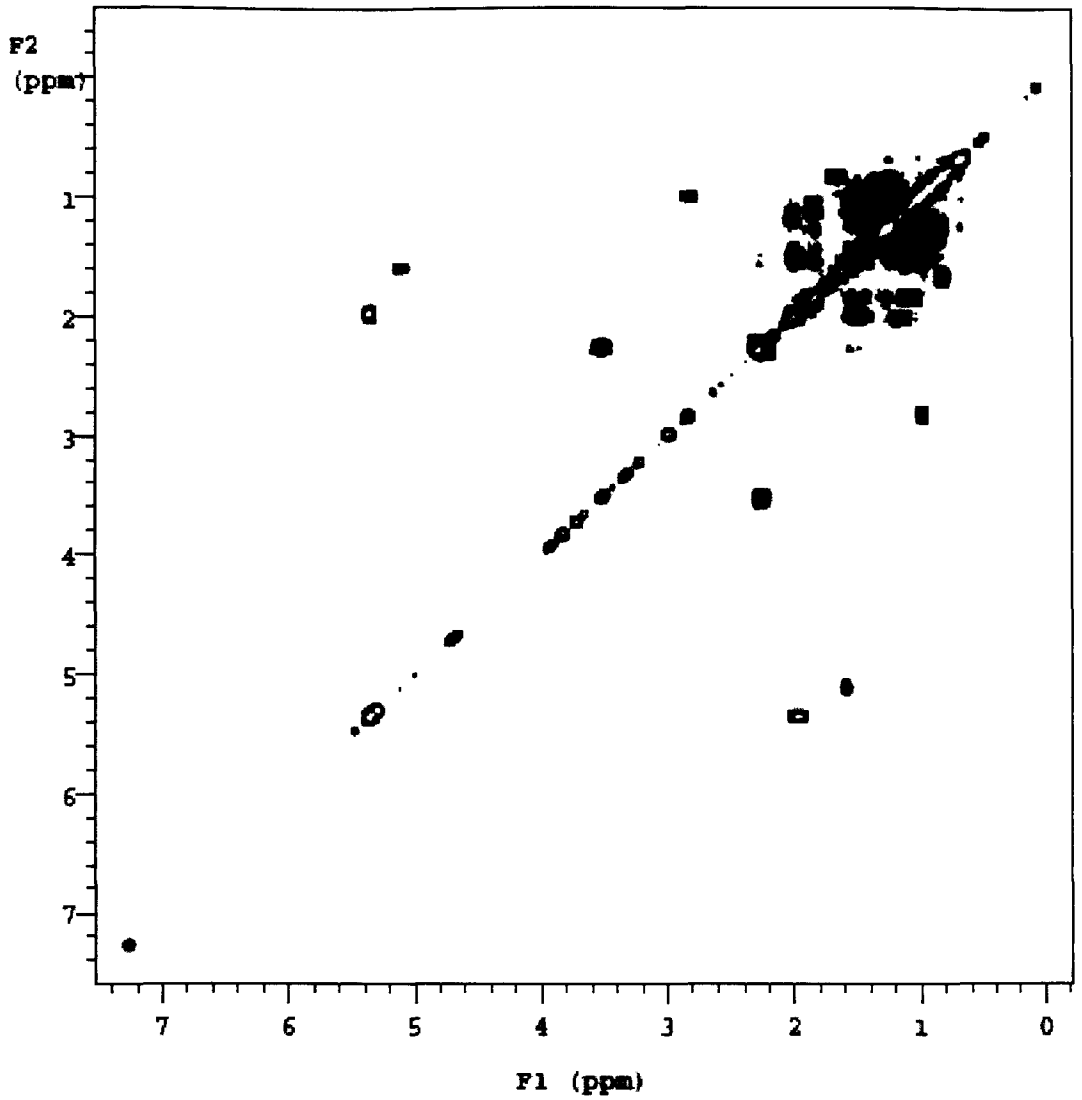

Figure D-5 Full COSY NMR of β-sitosterol isolated from fraction 6.

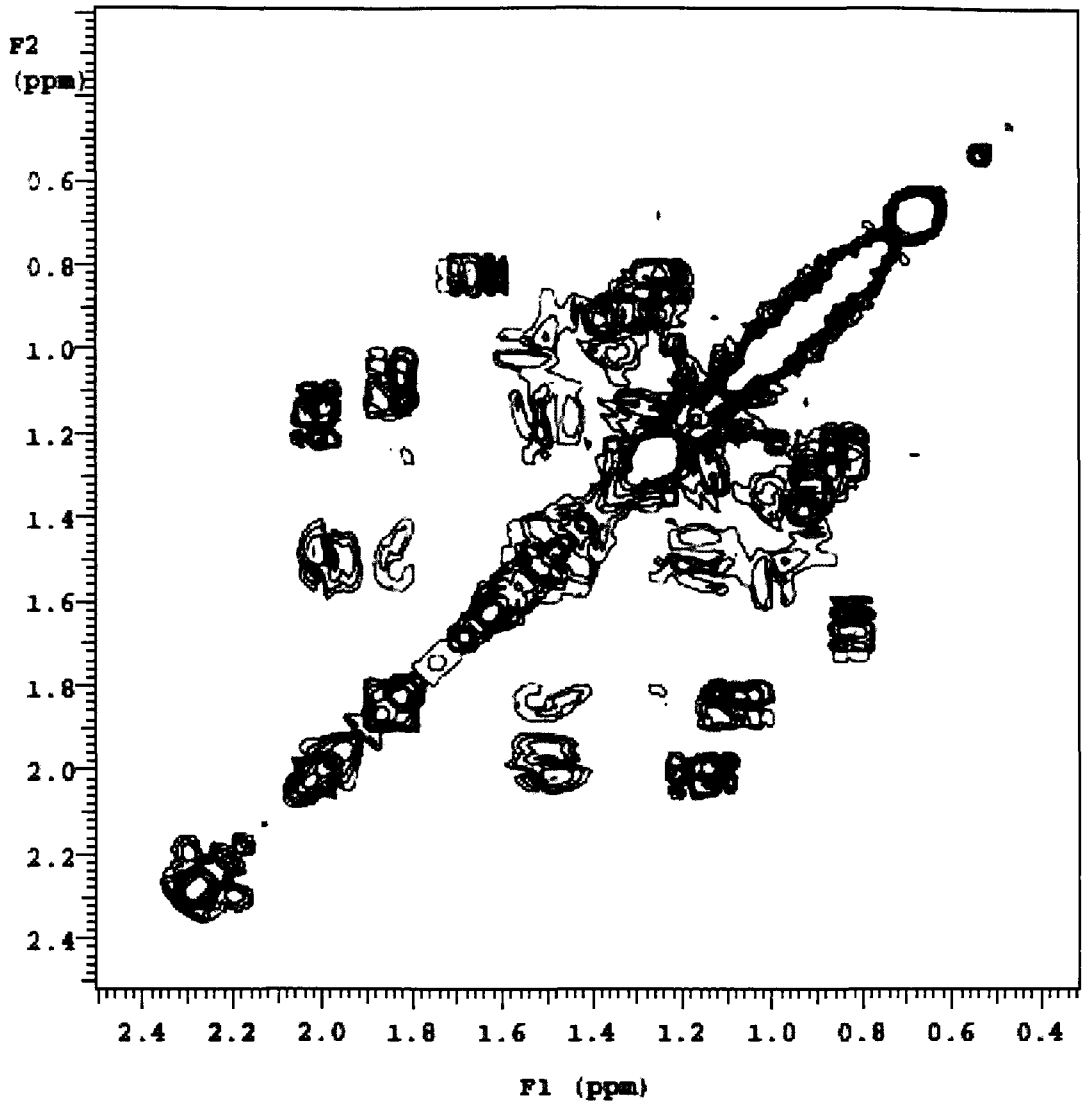

Figure D-6 Upfield COSY NMR of β-sitosterol isolated from fraction 6.

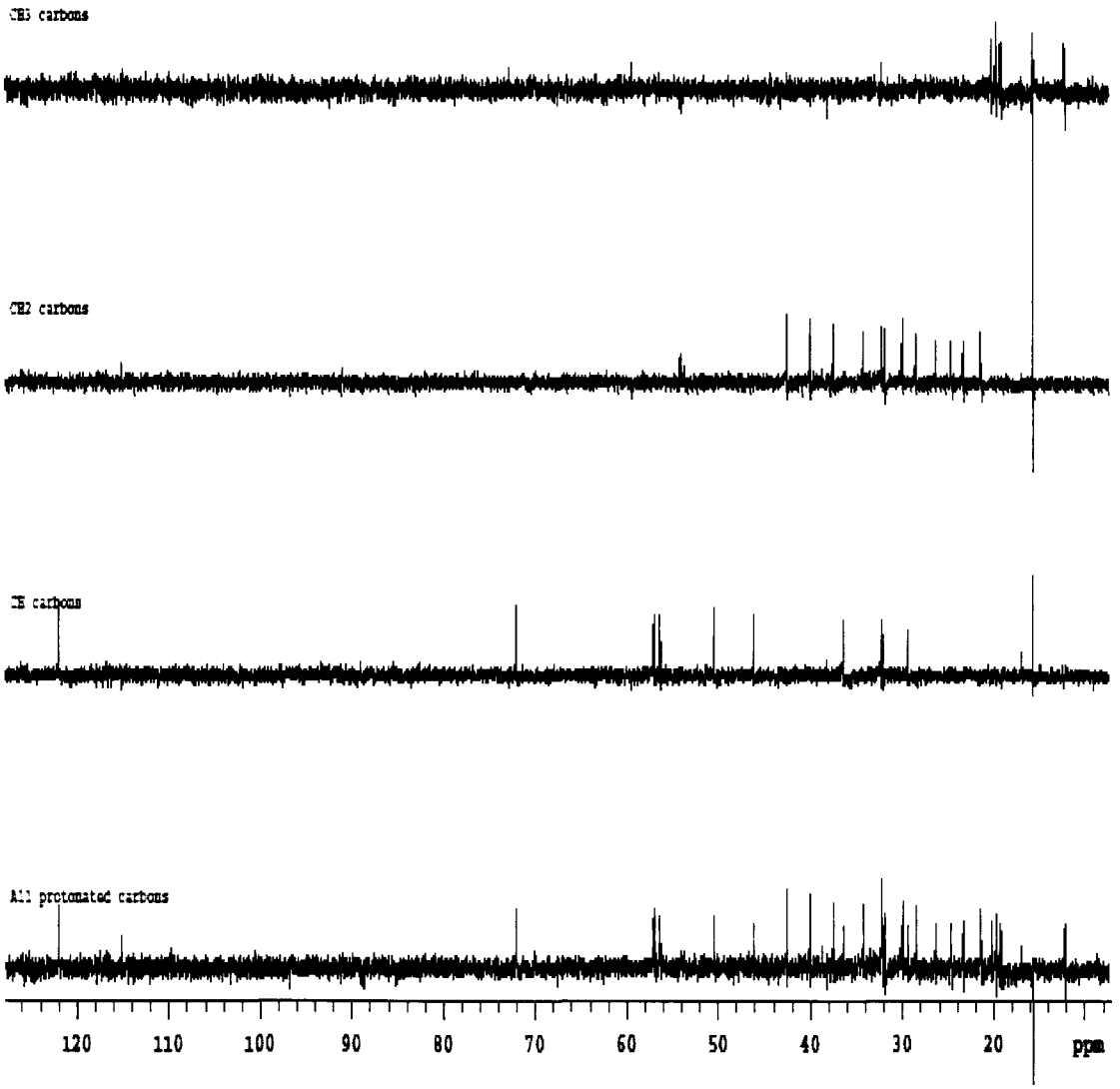

Figure D-7 DEPT NMR of β-sitosterol isolated from fraction 6.

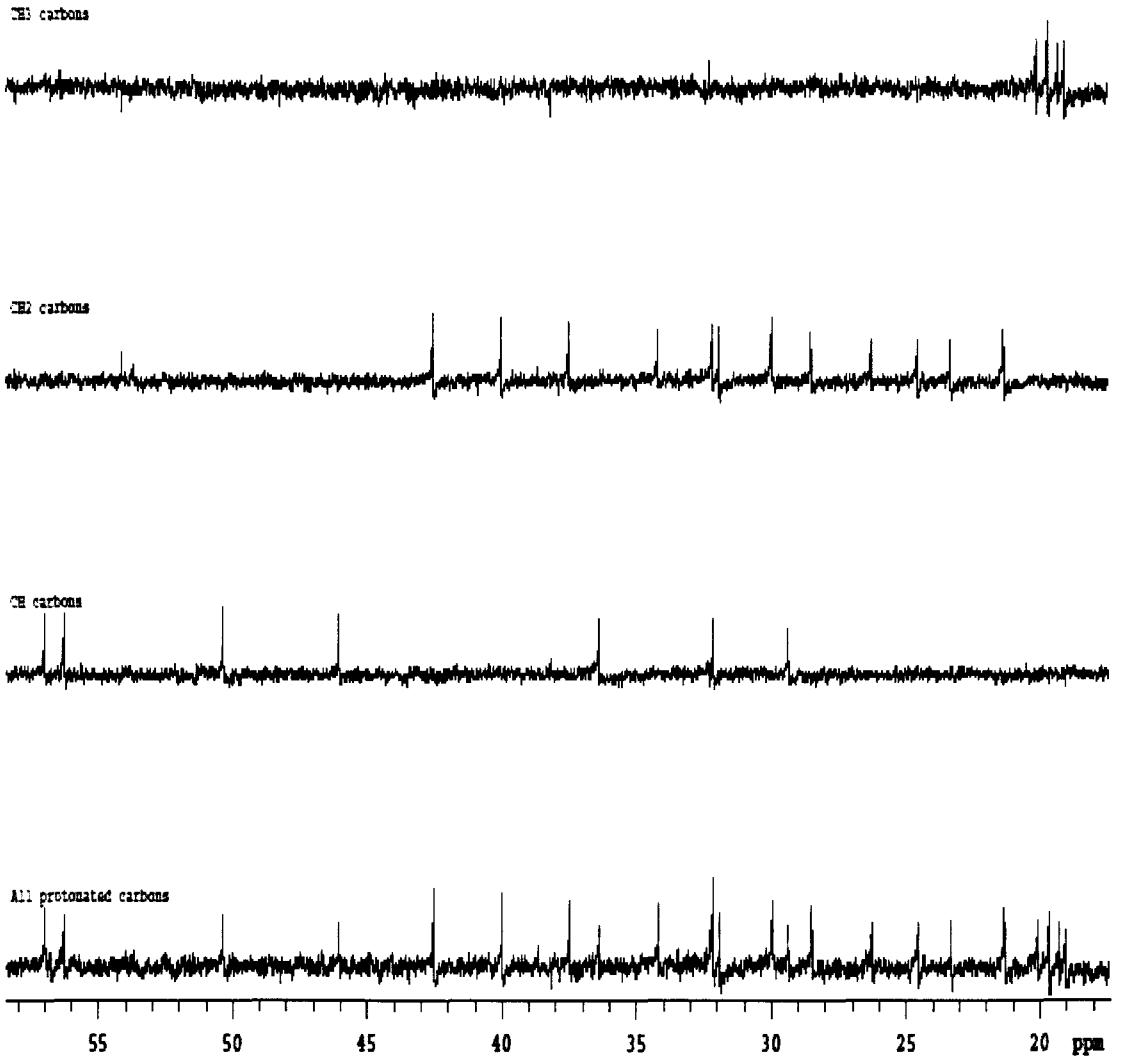

Figure D-8 Expanded DEPT NMR of β-sitosterol isolated from fraction 6.

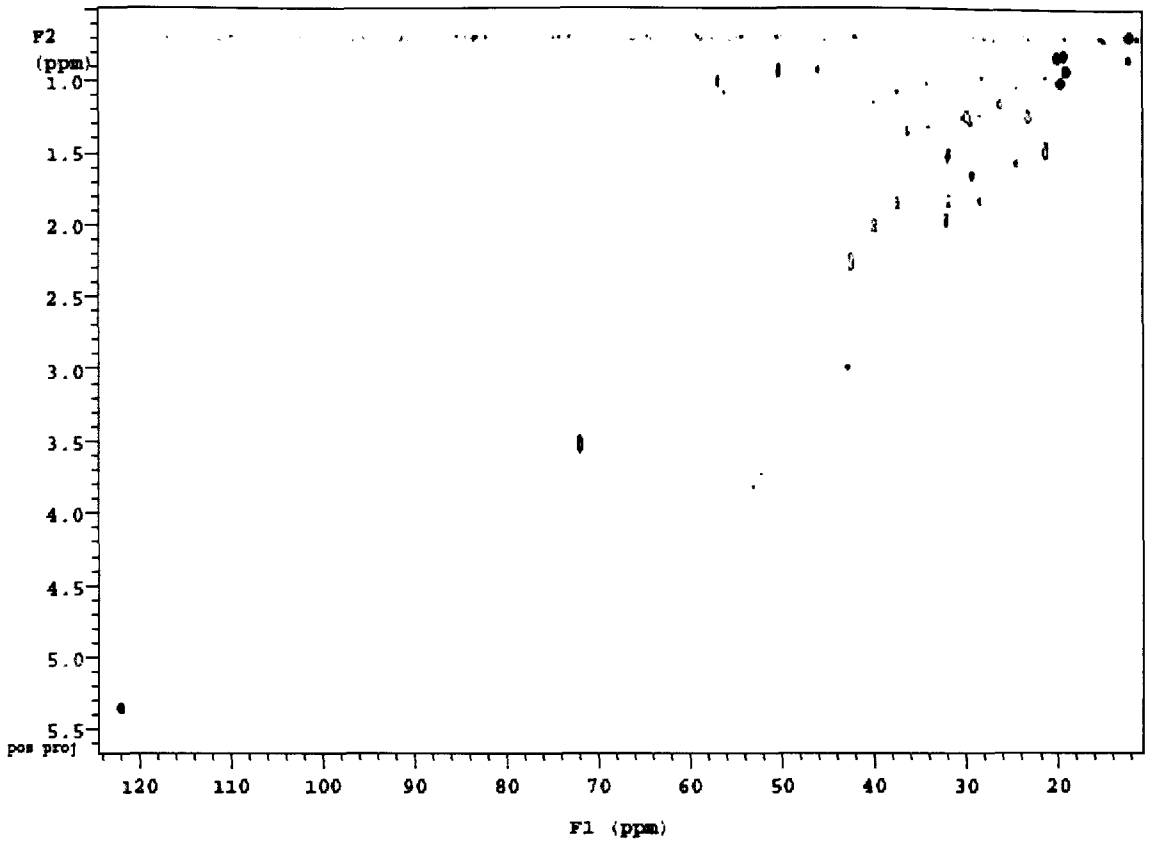

Figure D-9 Full gHSQC NMR of β-sitosterol isolated from fraction 6.

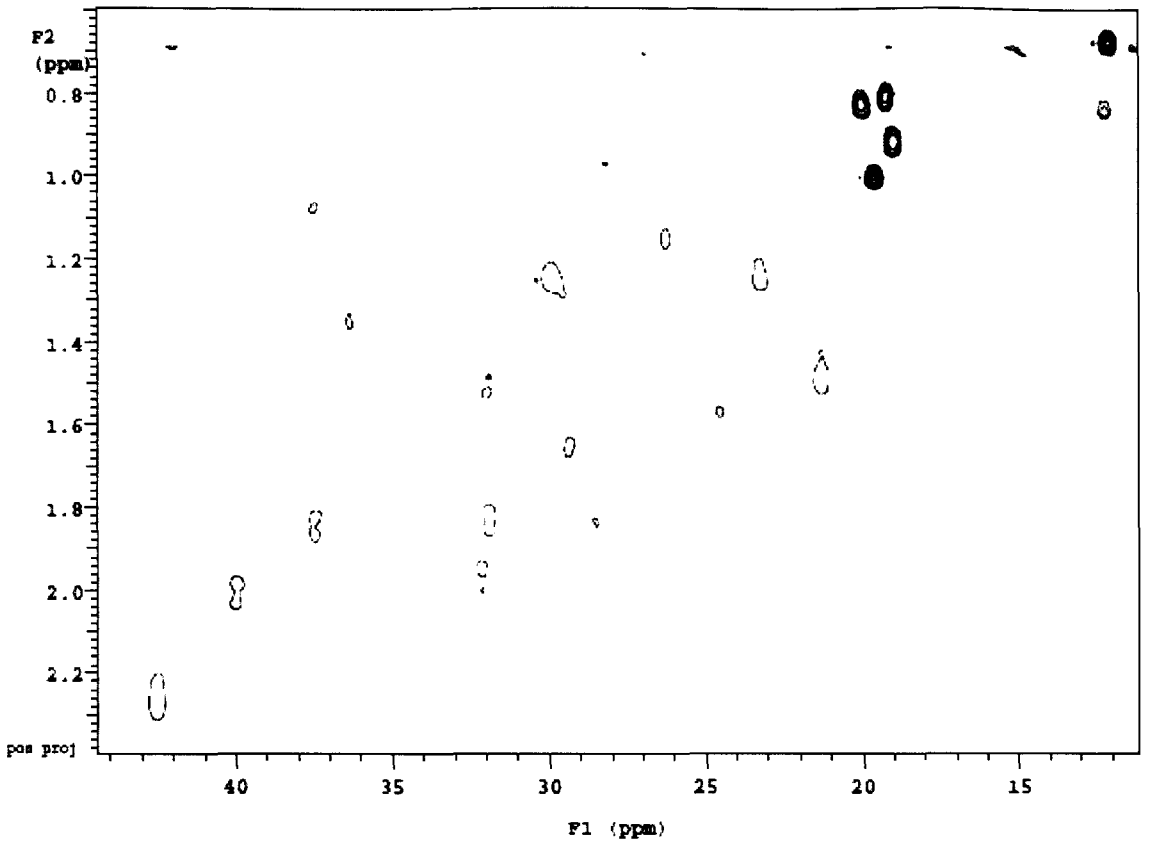

Figure D-10 Upfield gHSQC NMR of β-sitosterol isolated from fraction 6.

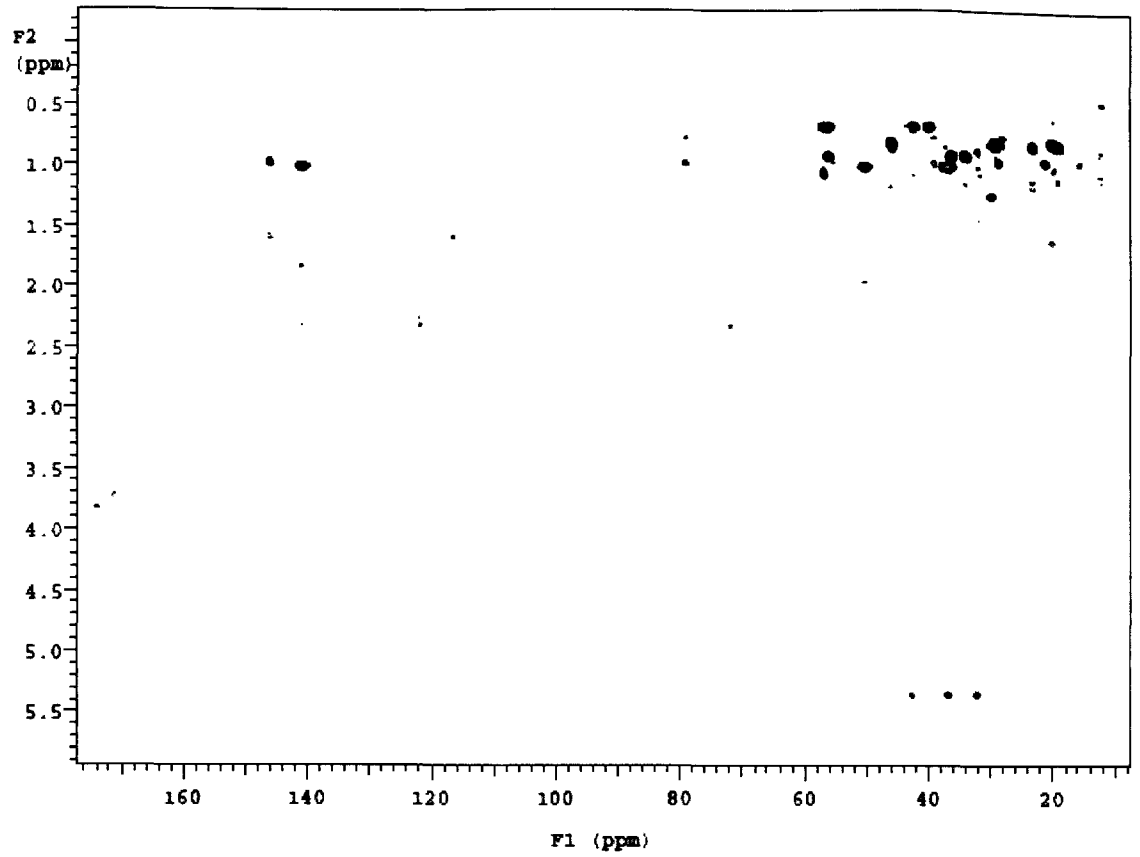

Figure D-11 Full gHMBC NMR of β-sitosterol isolated from fraction 6.

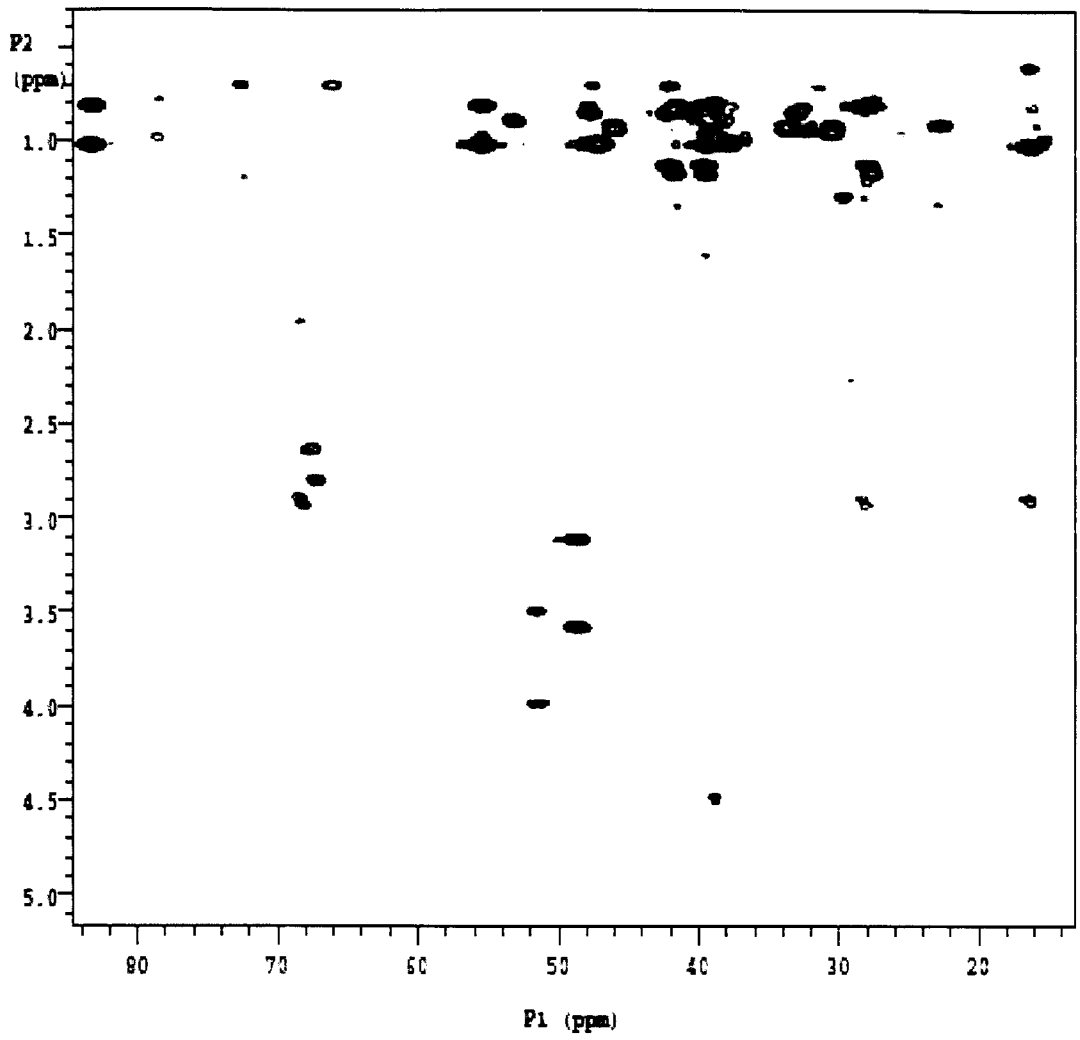

Figure D-12 Upfield gHMBC NMR of β-sitosterol isolated from fraction 6.

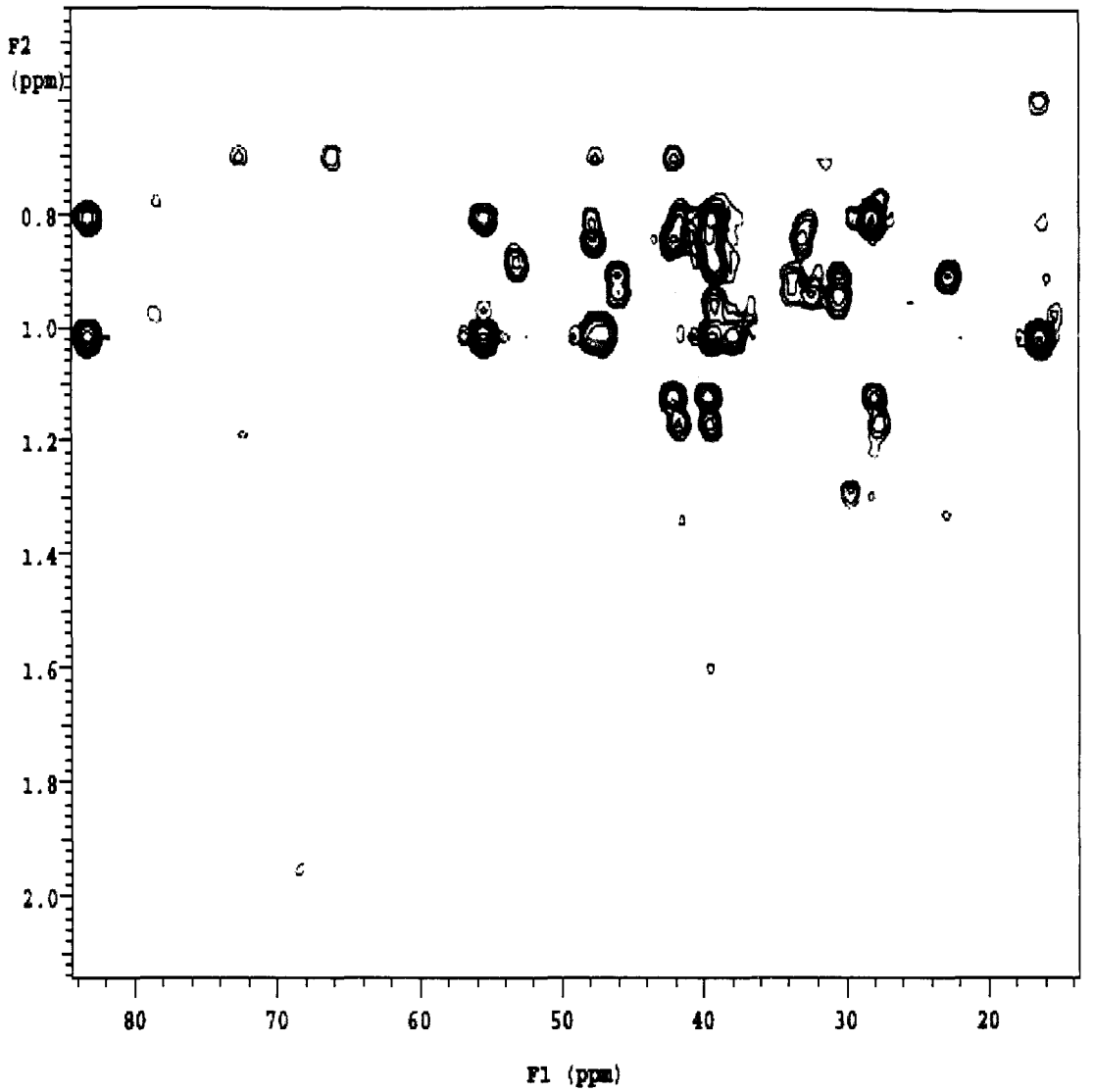

Figure D-13 Upfield gHMBC of β-sitosterol focused on overlapping regions.

Appendix E

Supplementary NMR spectra for ursolic acid

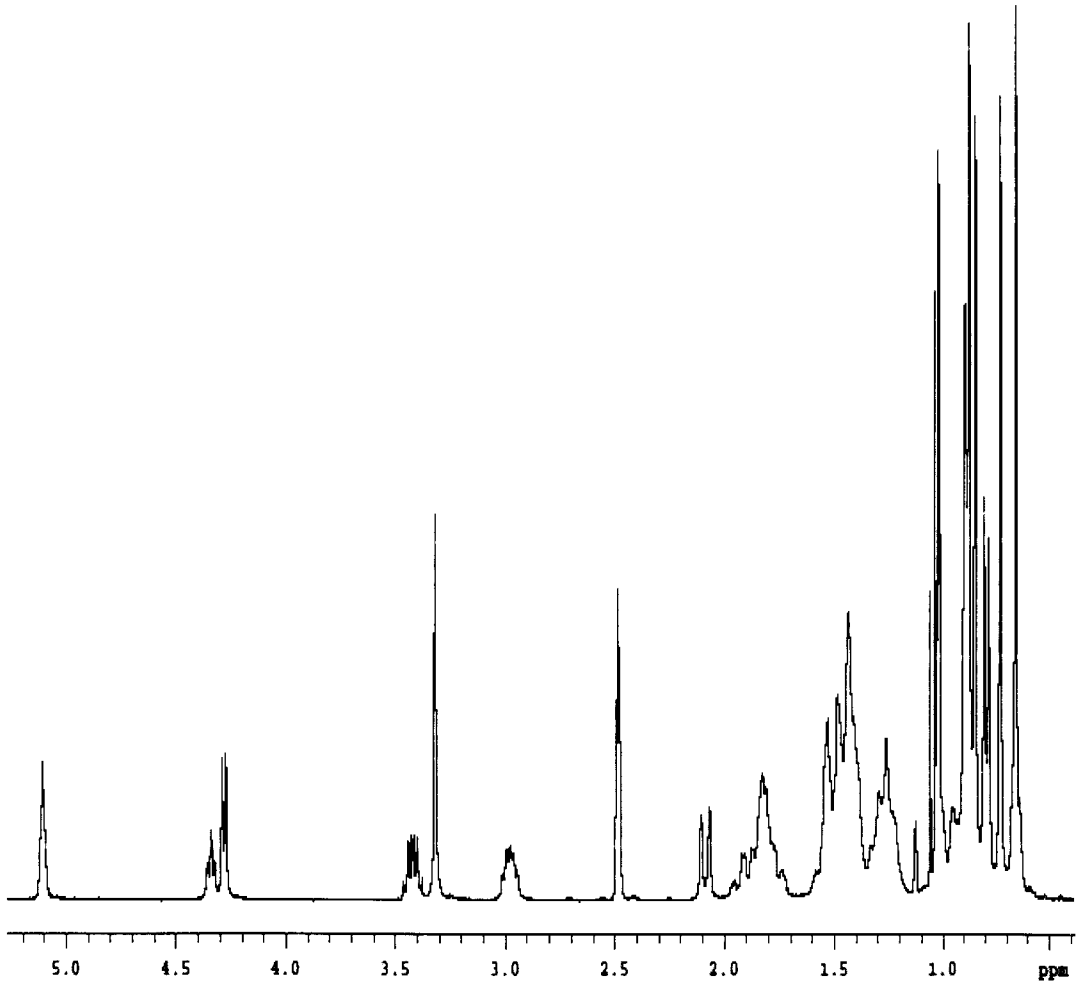

Figure E-1 Full ^{1}H NMR of ursolic acid isolated from fraction 8. Omitting the carboxylic acid at 11.9 ppm for clarity in the upfield region.

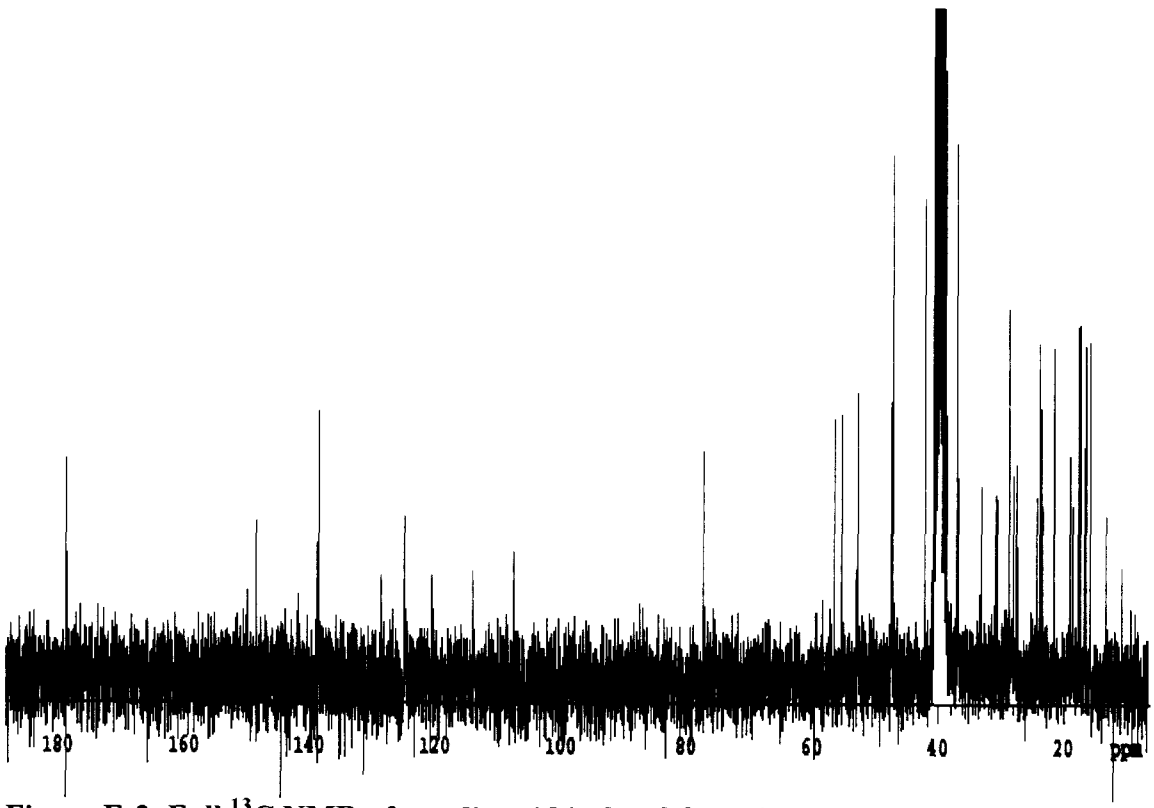

Figure E-2 Full ^{13}C NMR of ursolic acid isolated from fraction 8.

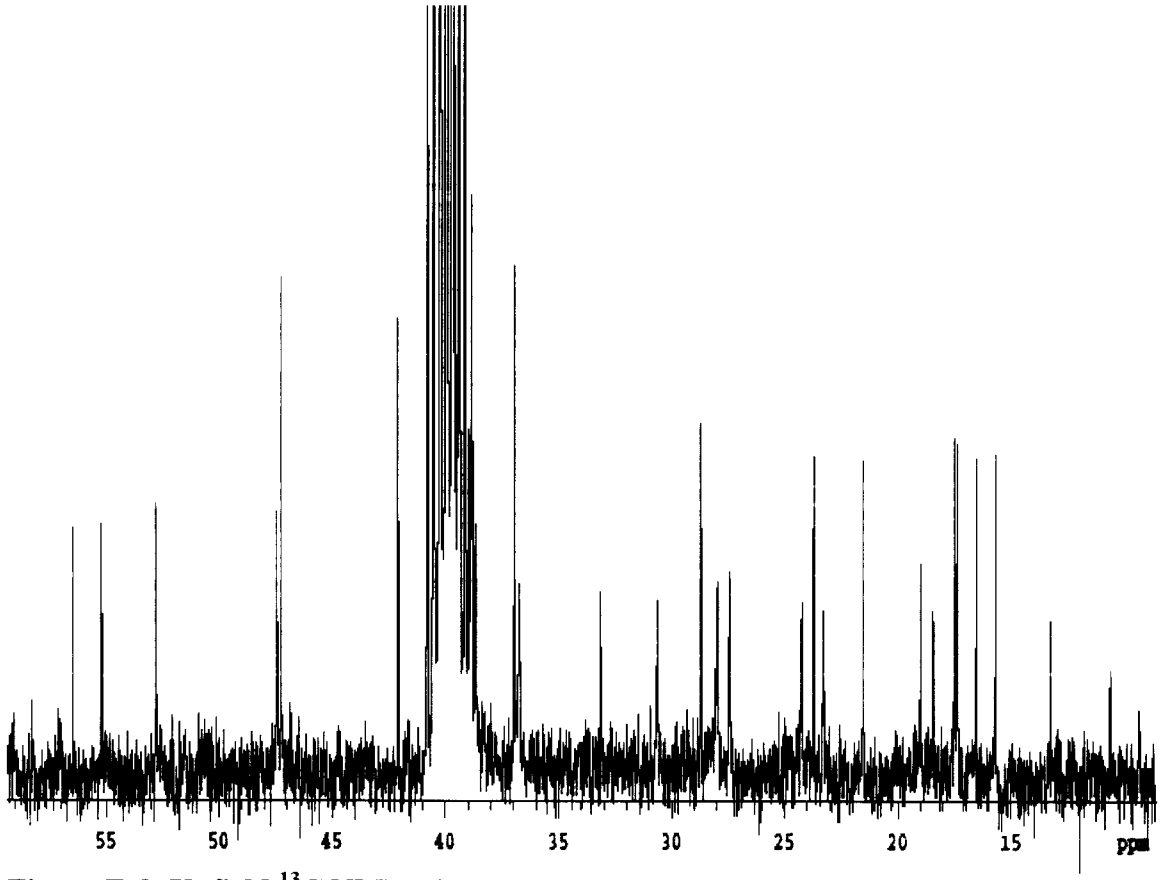

Figure E-3 Upfield ^{13}C NMR of ursolic acid isolated from fraction 8.

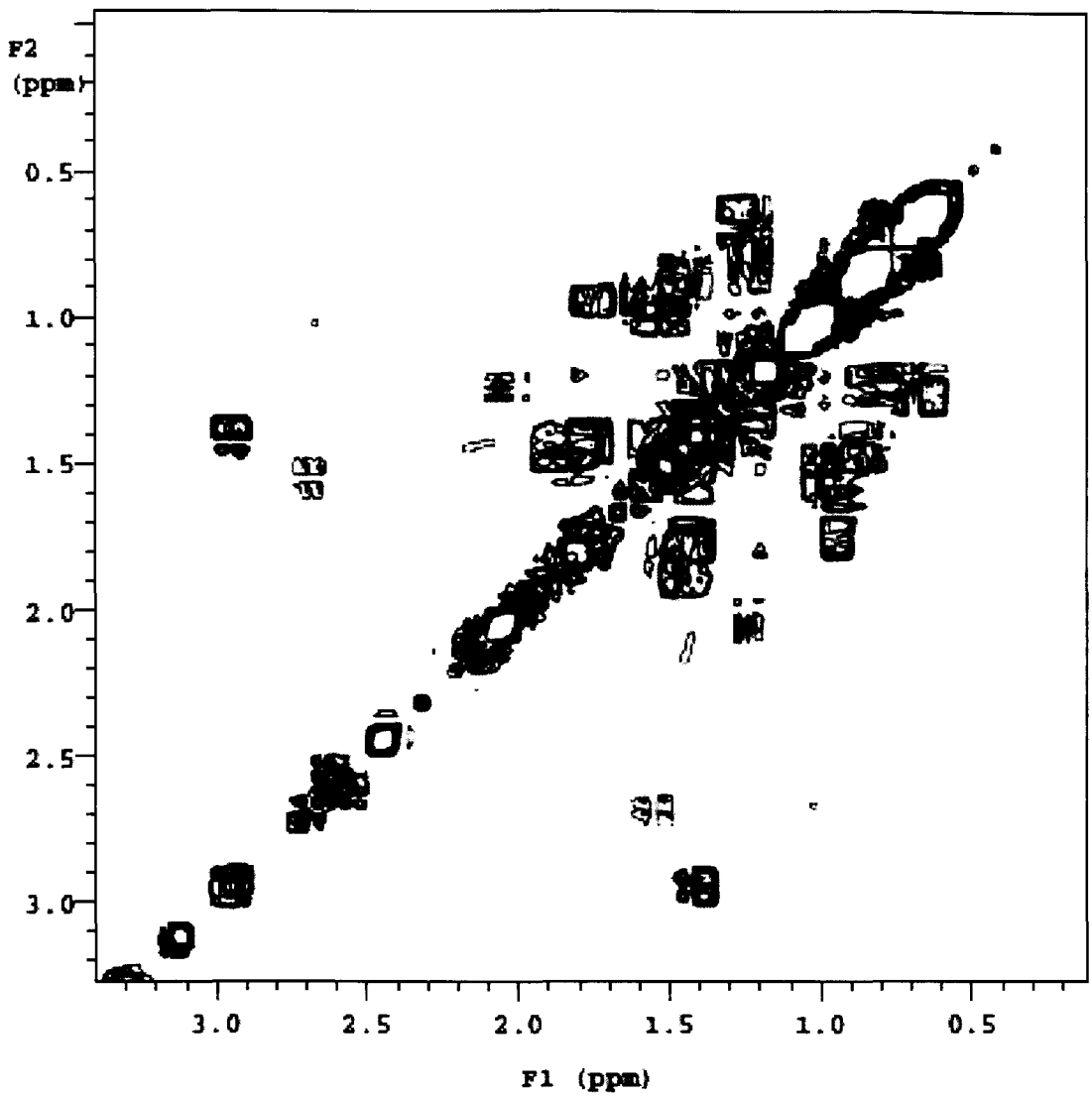

Figure E-4 Relevant COSY NMR of ursolic acid isolated from fraction 8.

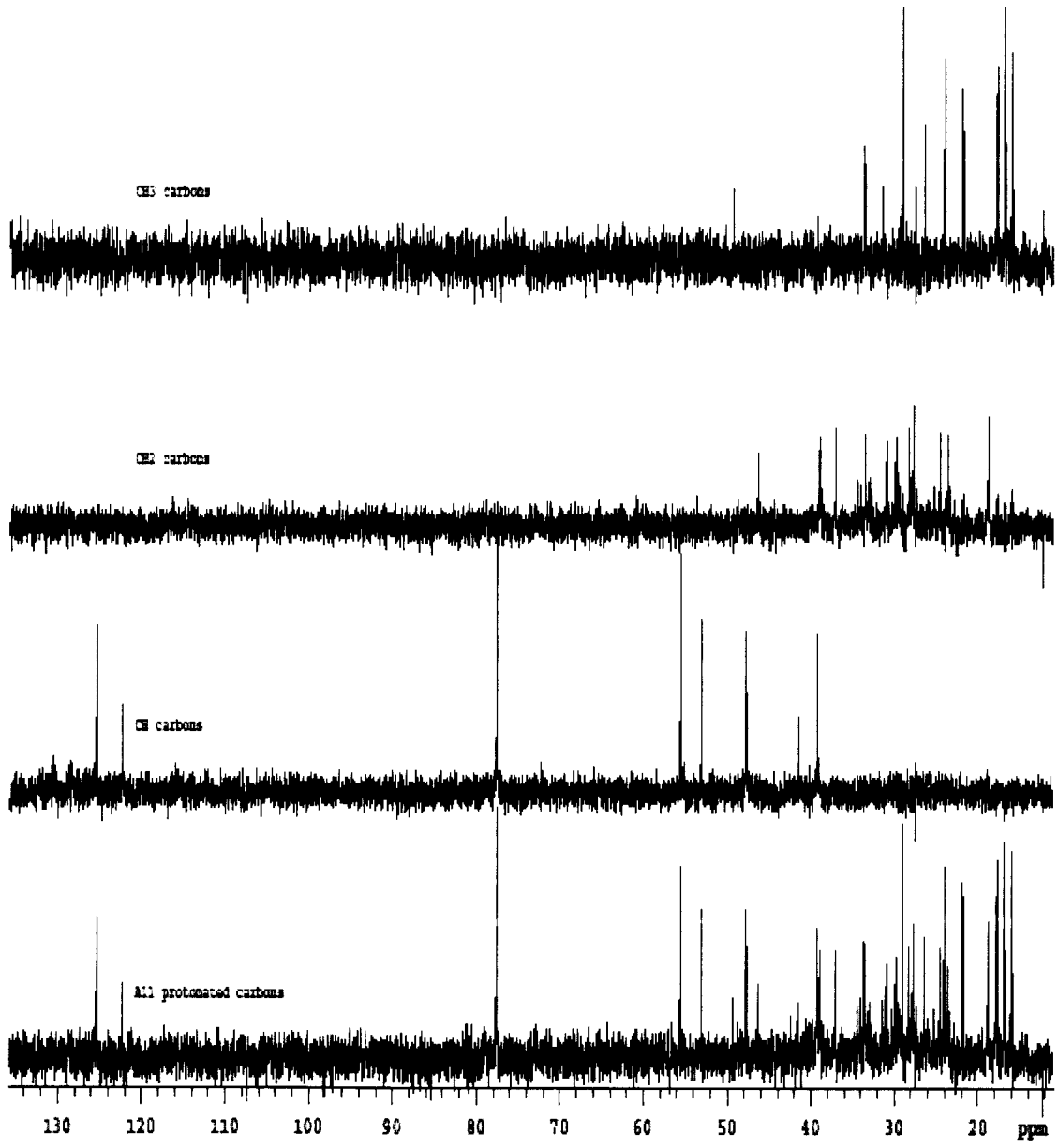

Figure E-5 DEPT NMR of ursolic acid isolated from fraction 8.

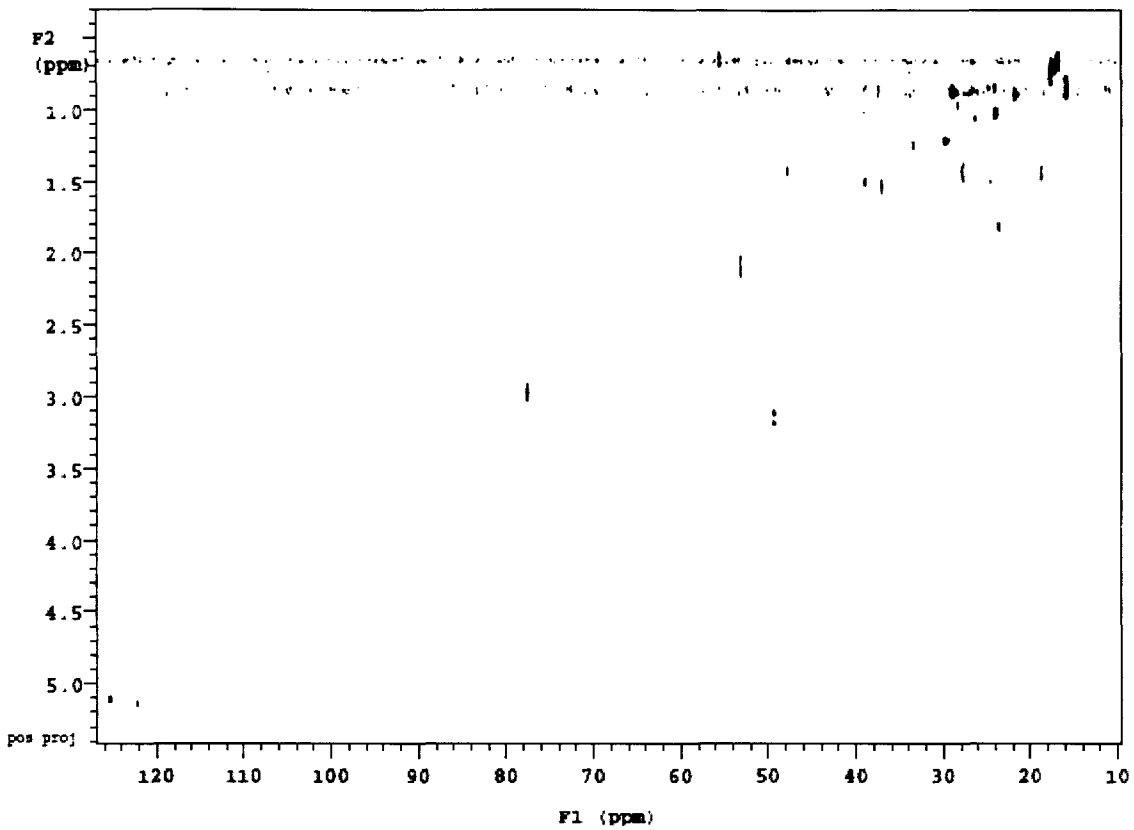

Figure E-6 Full gHSQC NMR of ursolic acid isolated from fraction 8.

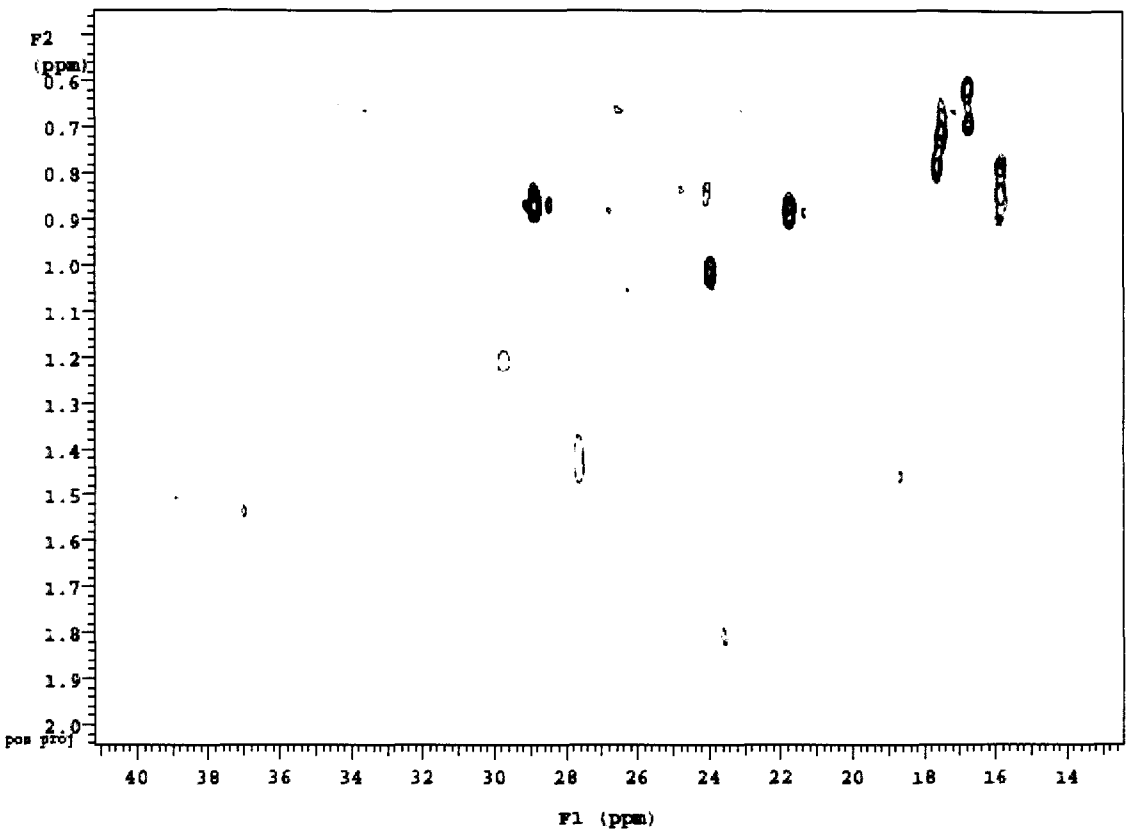

Figure E-7 Upfield region of gHSQC NMR of ursolic acid isolated from fraction 8.

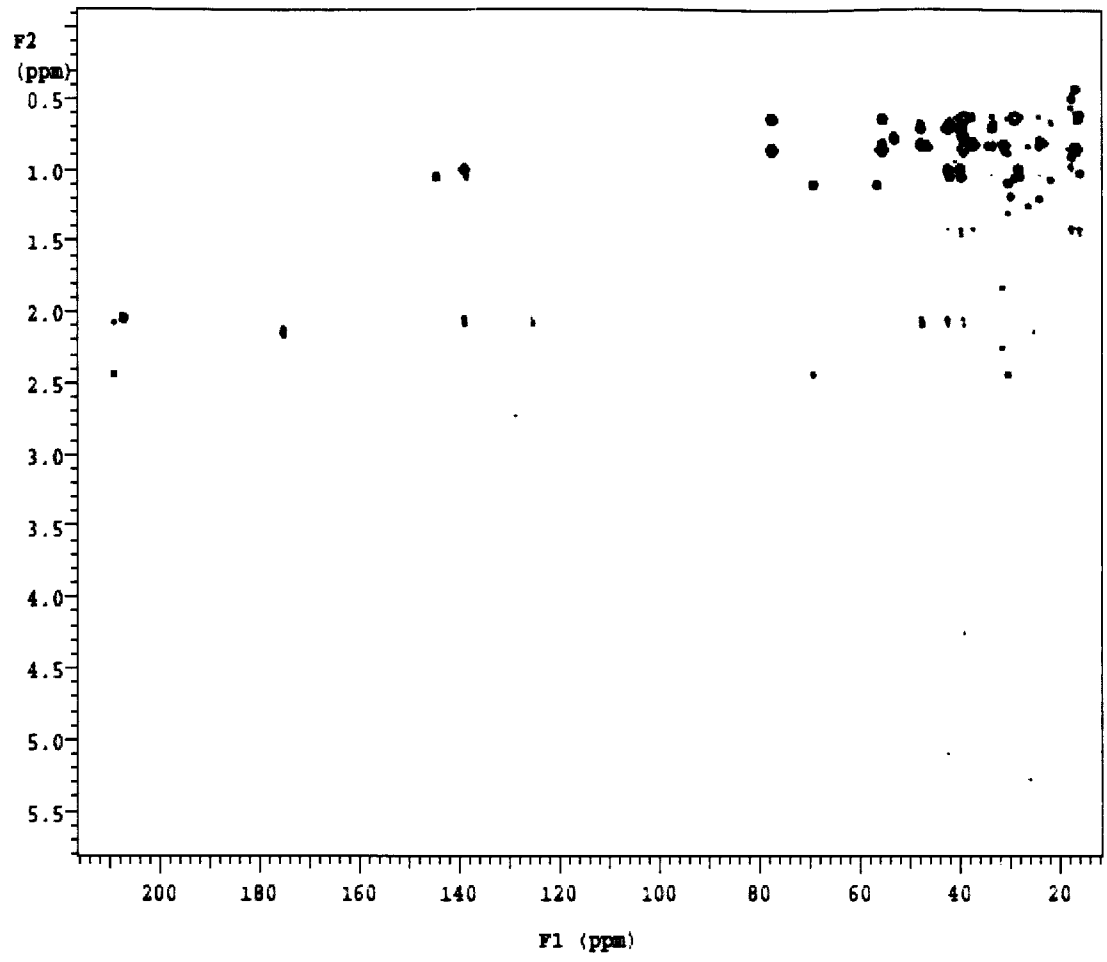

Figure E-8 Full gHMBC of ursolic acid isolated from fraction 8.

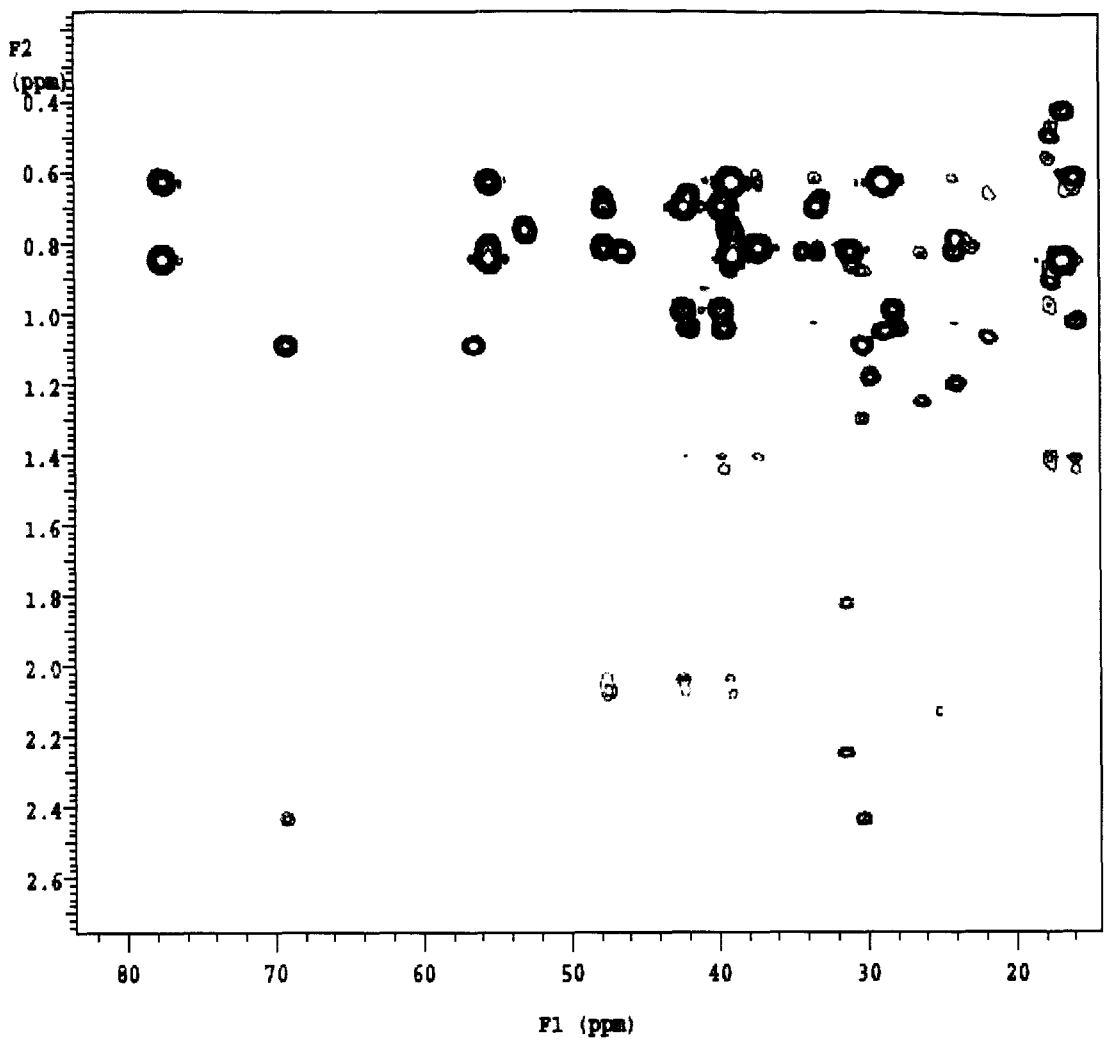

Figure E-9 Upfield region of gHMBC of ursolic acid isolated from fraction 8.

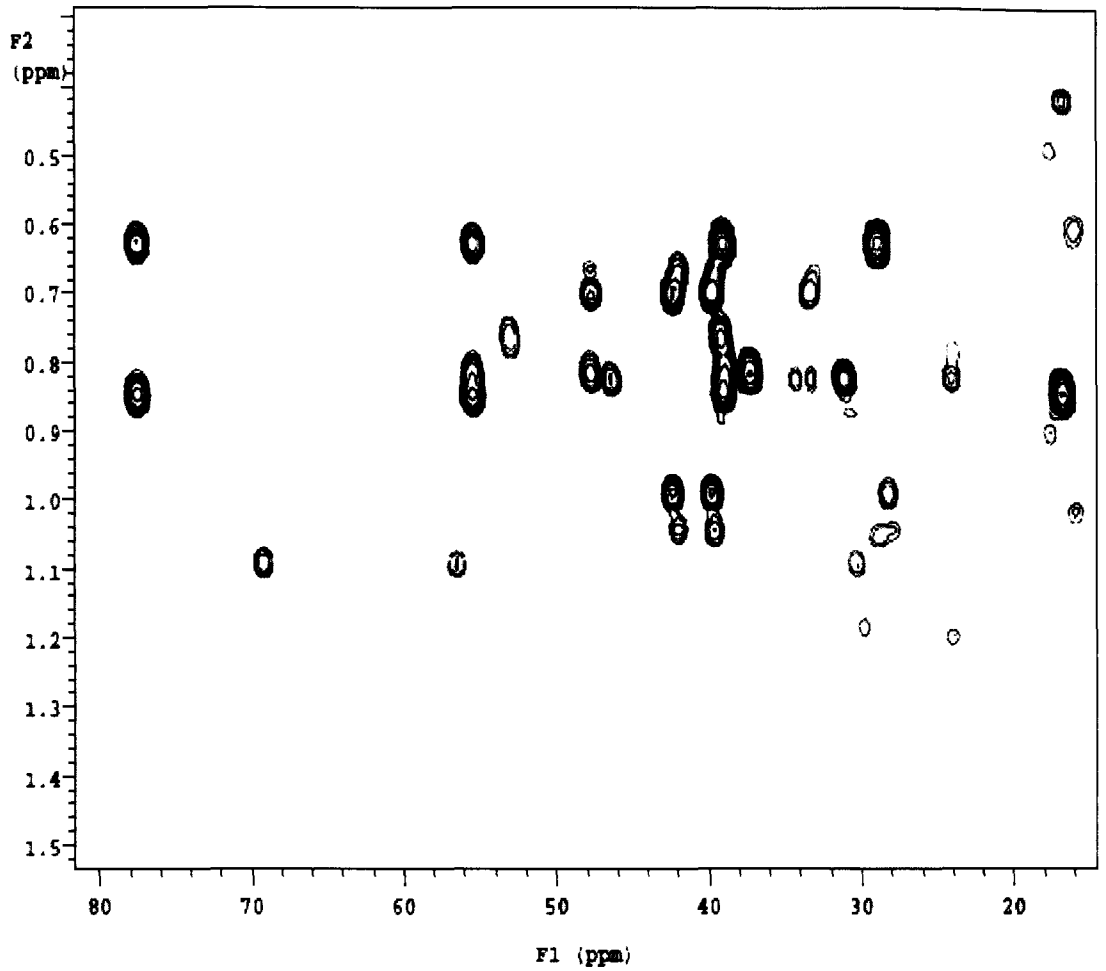

Figure E-10 Second upfield region of ursolic acid isolated from fraction 8, showing overlapping sections.

Appendix F

Supplementary NMR spectra for DPG and blueberric acid

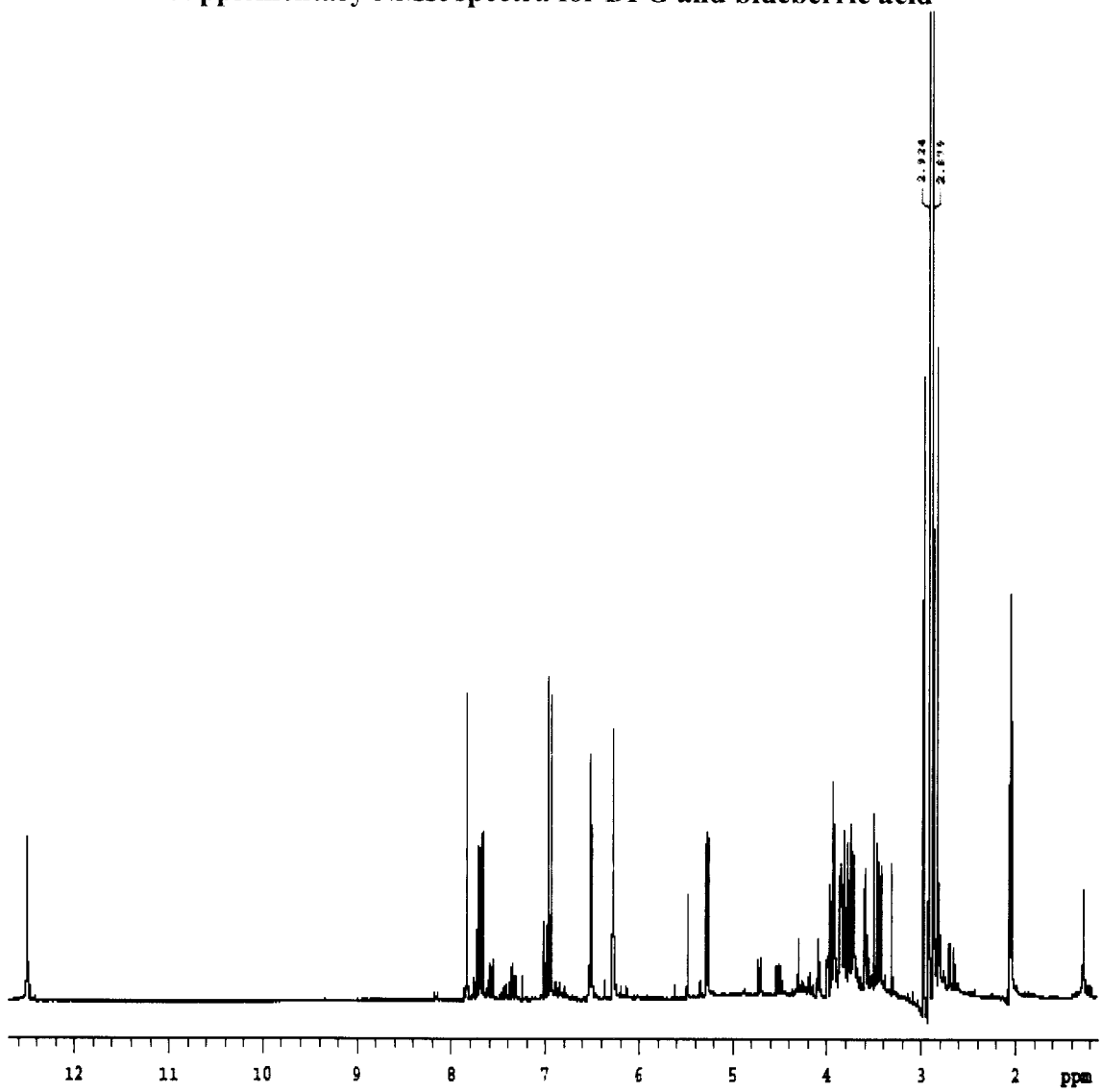

Figure F-1 ^{1}H NMR of DPG and blueberric acid isolated from fraction 28,8.

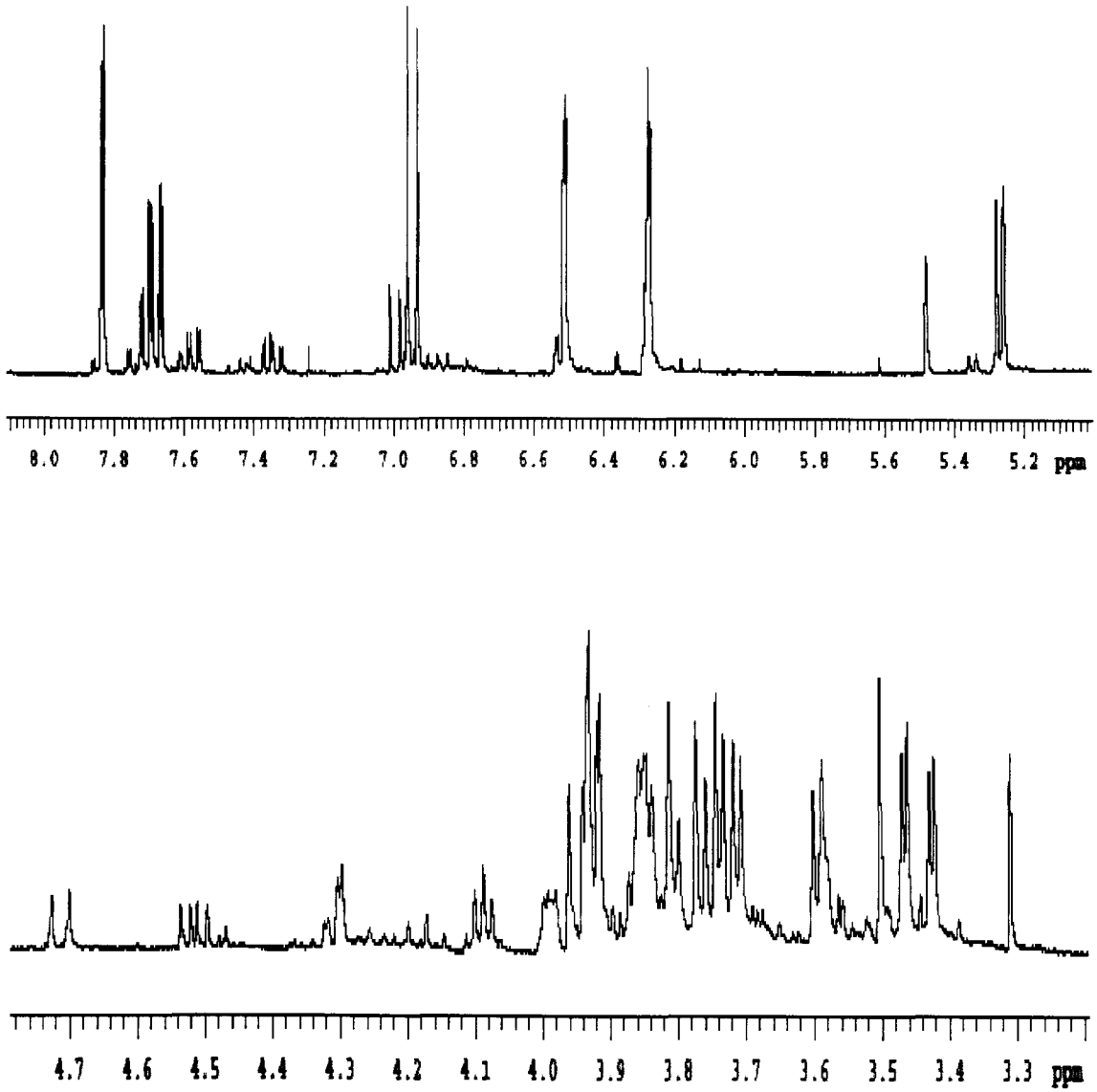

Figure F-2 Downfield and upfield sections of ^{1}H NMR of DPG and blueberric acid isolated from fraction 28,8.

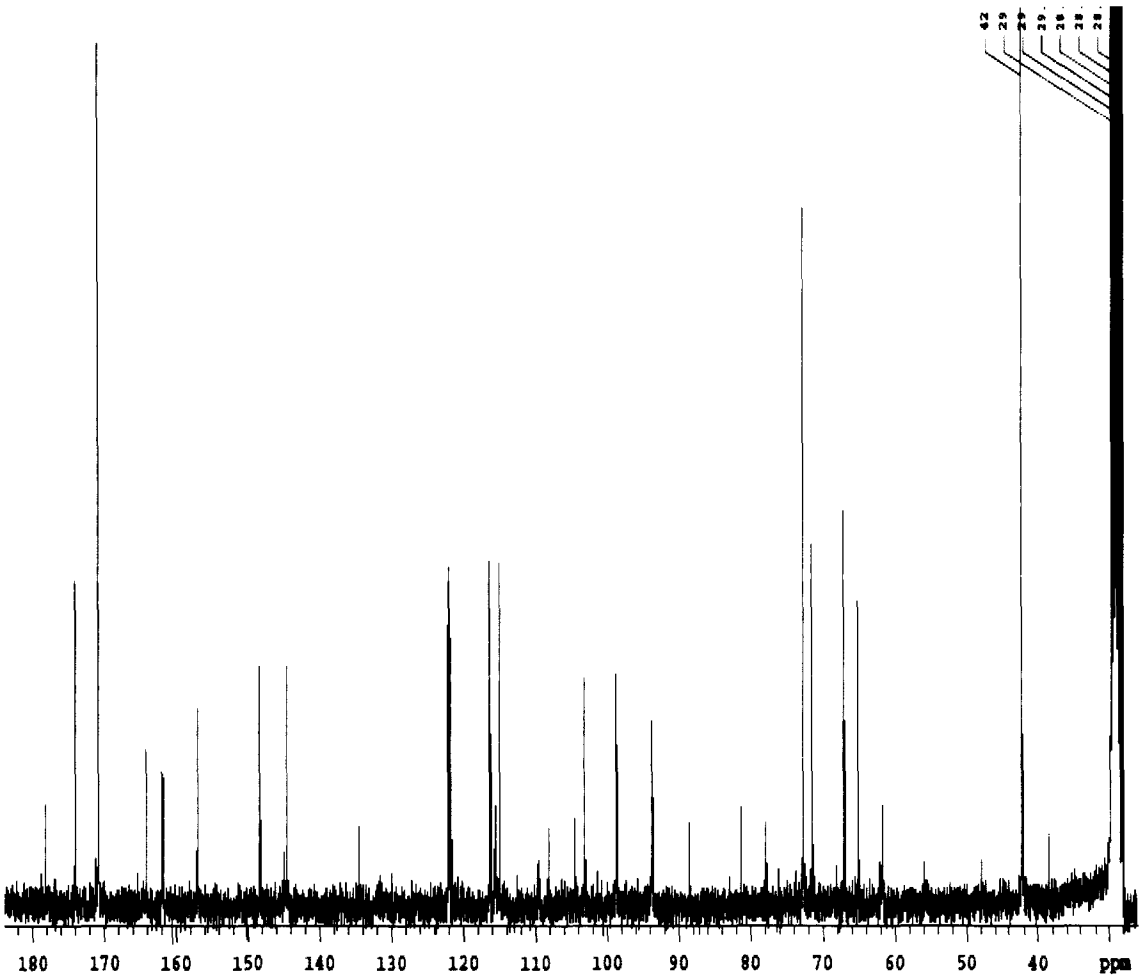

Figure F-3 Whole ^{13}C NMR of DPG and blueberric acid.

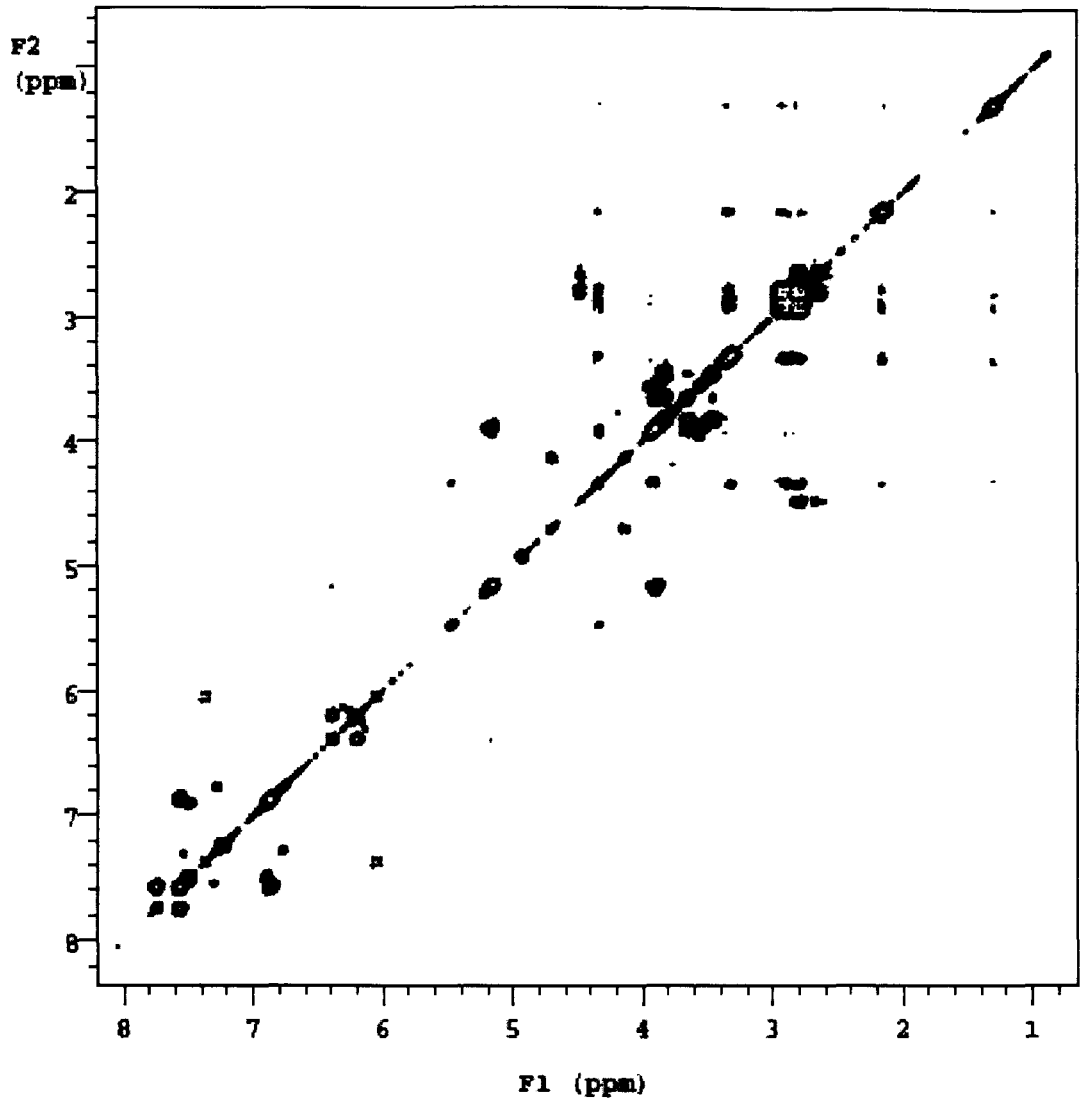

Figure F-4 COSY NMR of DPG and blueberric acid isolated from fraction 28,8.

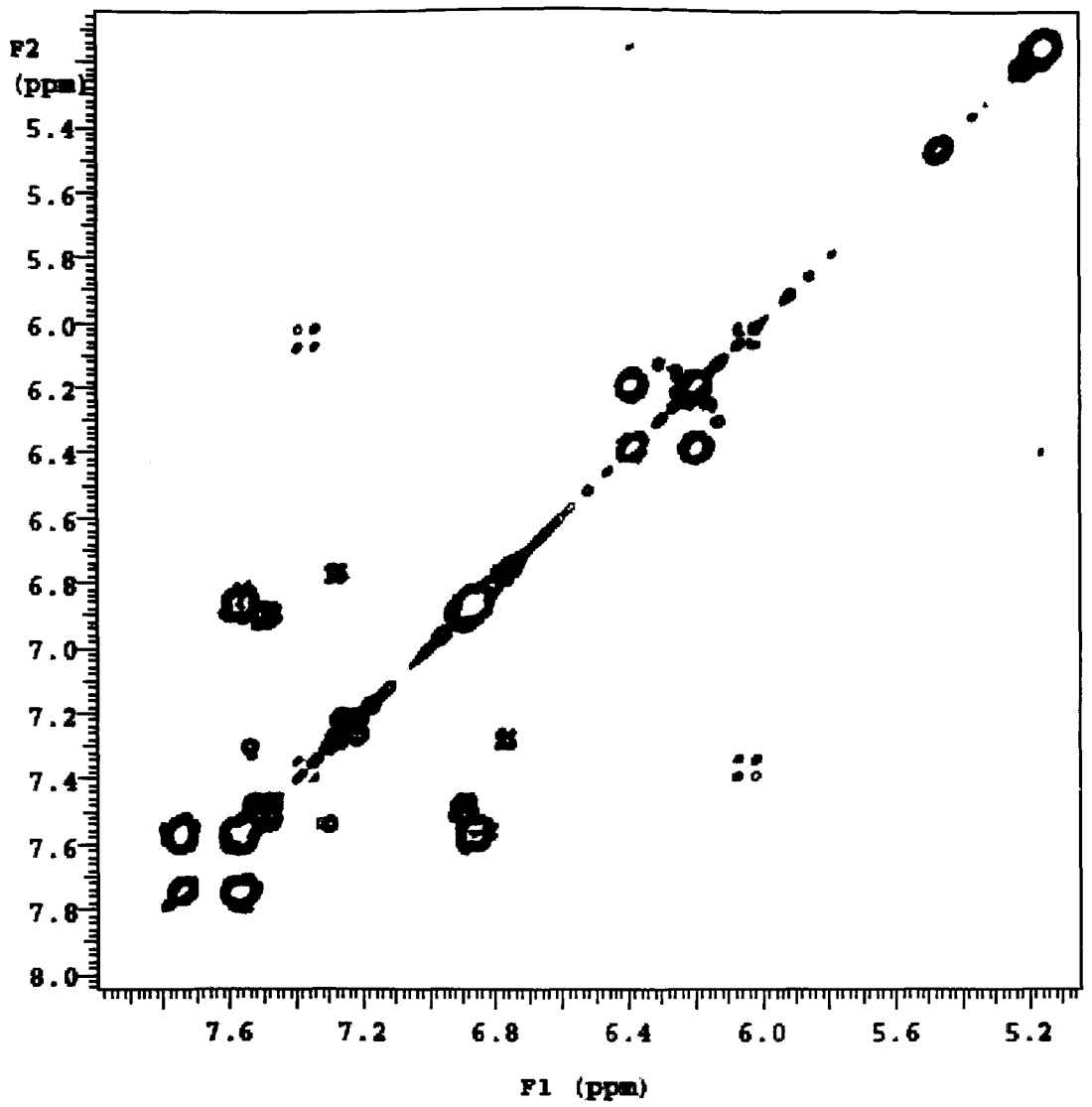

Figure F-5 Downfield region of COSY NMR of DPG and blueberric acid isolated from fraction 28,8.

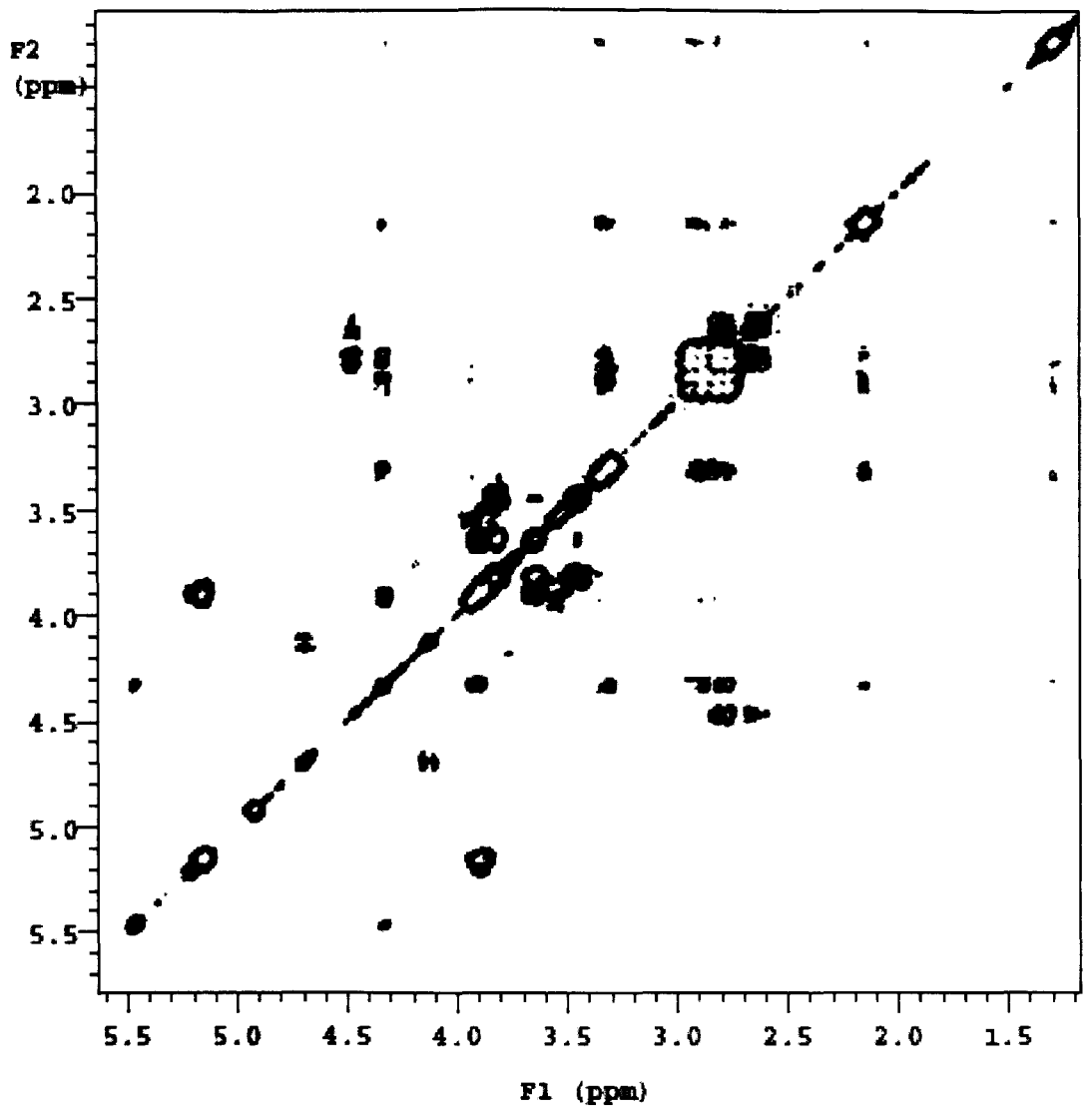

Figure F-6 Upfield region of COSY NMR of DPG and blueberric acid isolated from fraction 28,8.

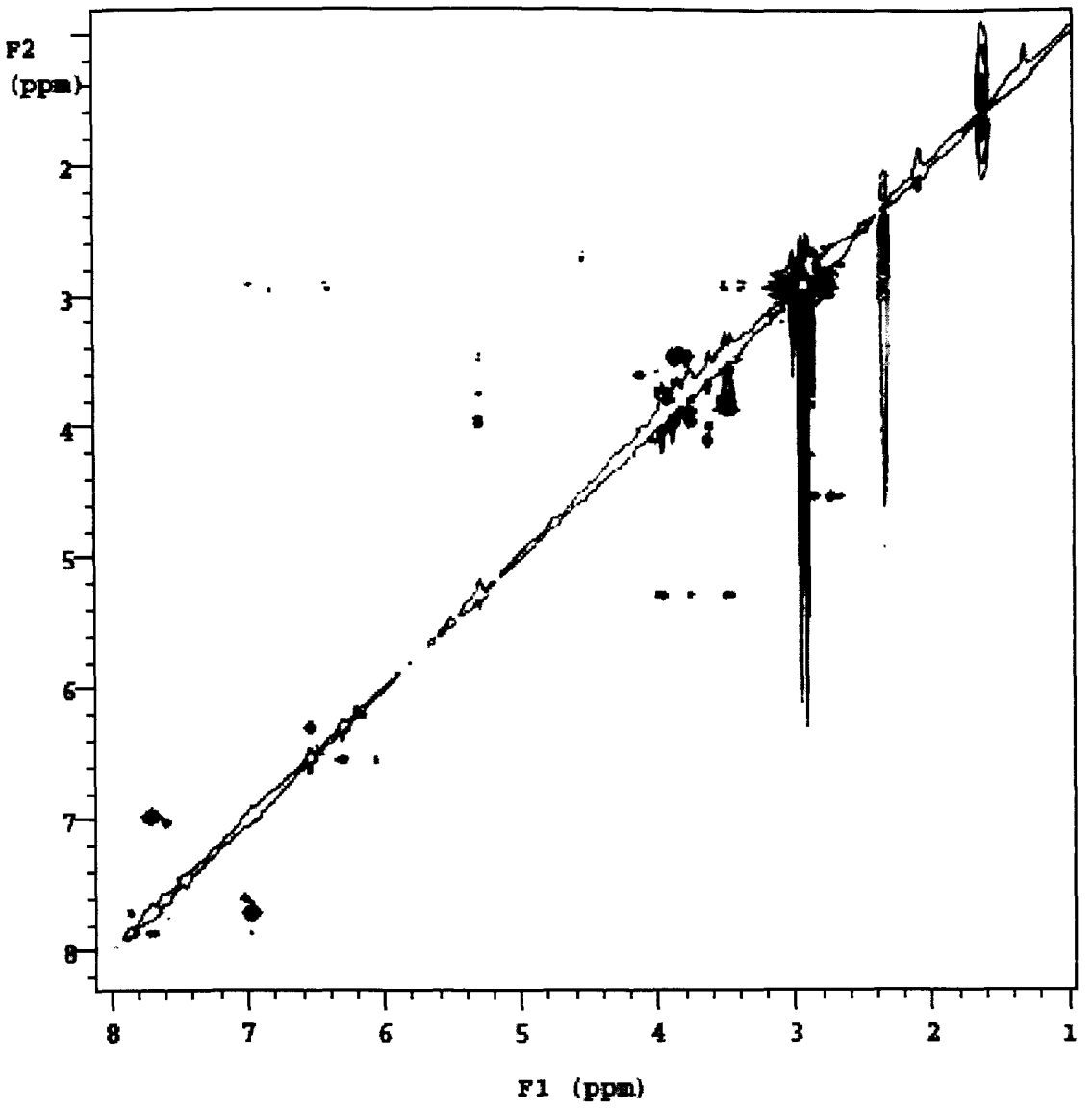

Figure F-7 Full NOESY NMR of DPG and blueberric acid isolated from fraction 28,8.

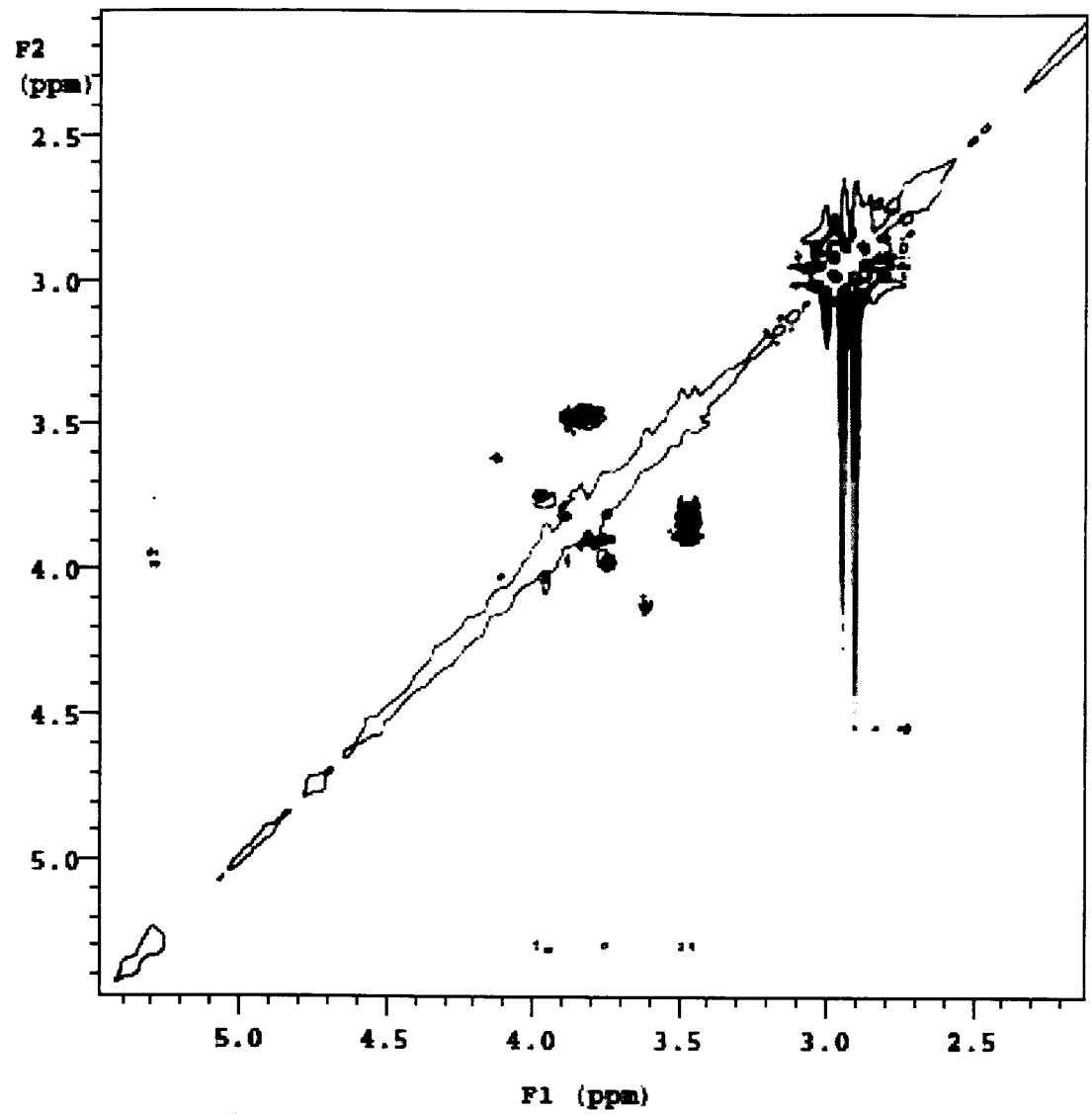

Figure F-8 Upfield region of NOESY NMR of DPG and blueberric acid isolated from fraction 28,8.

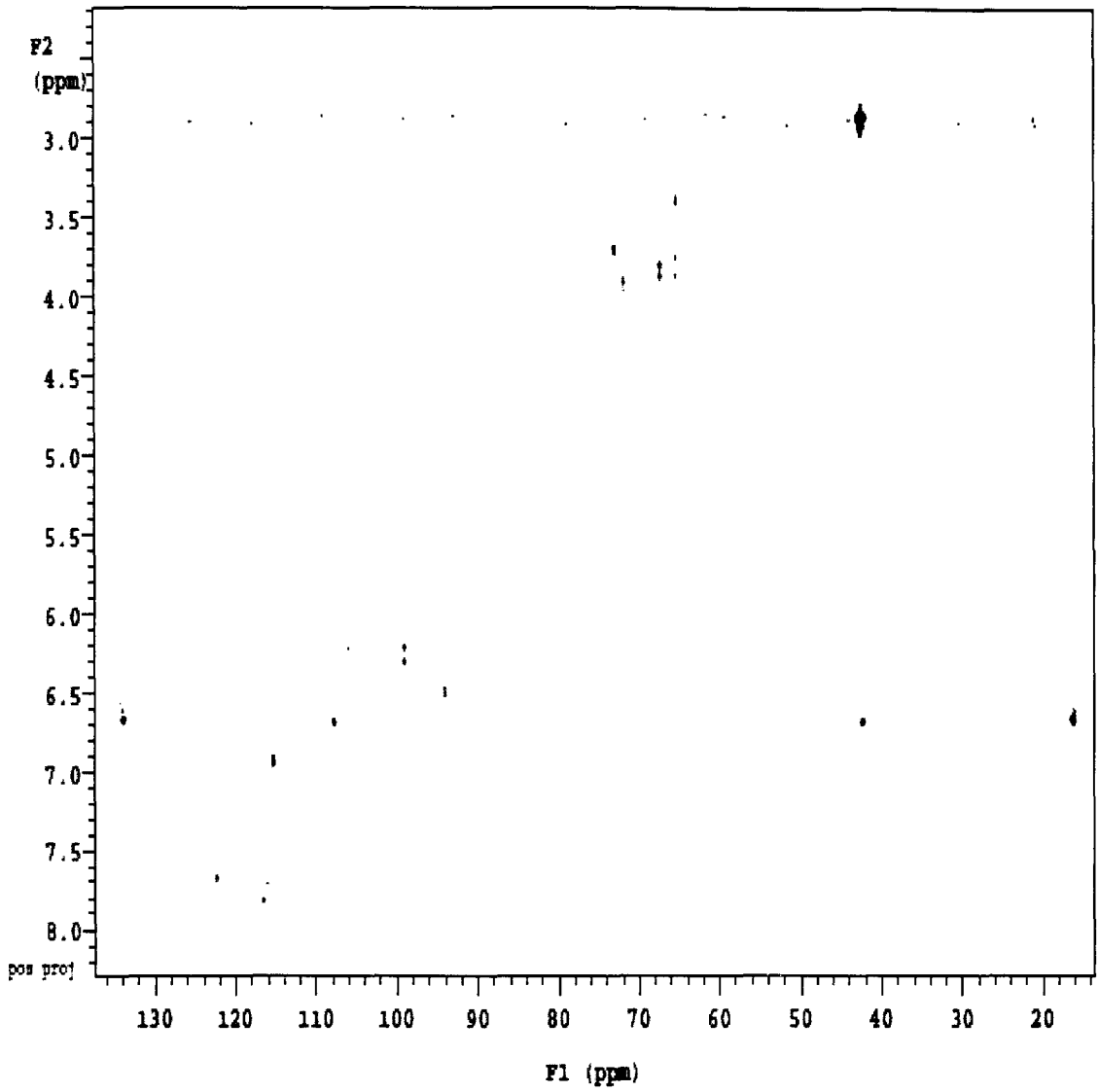

Figure F-9 Full gHSQC NMR of DPG and blueberric acid.

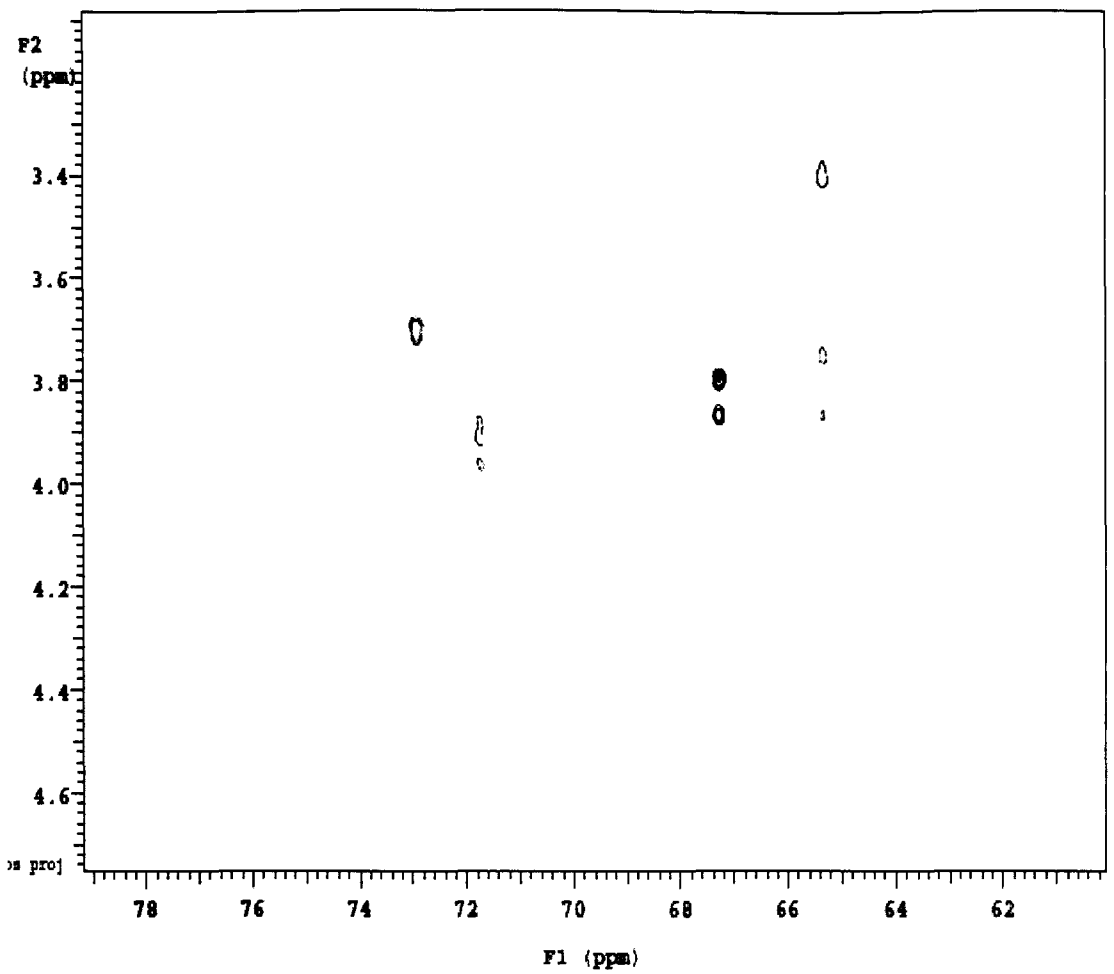

Figure F-10 Upfield gHSQC NMR of DPG and blueberric acid isolated from fraction 28,8.

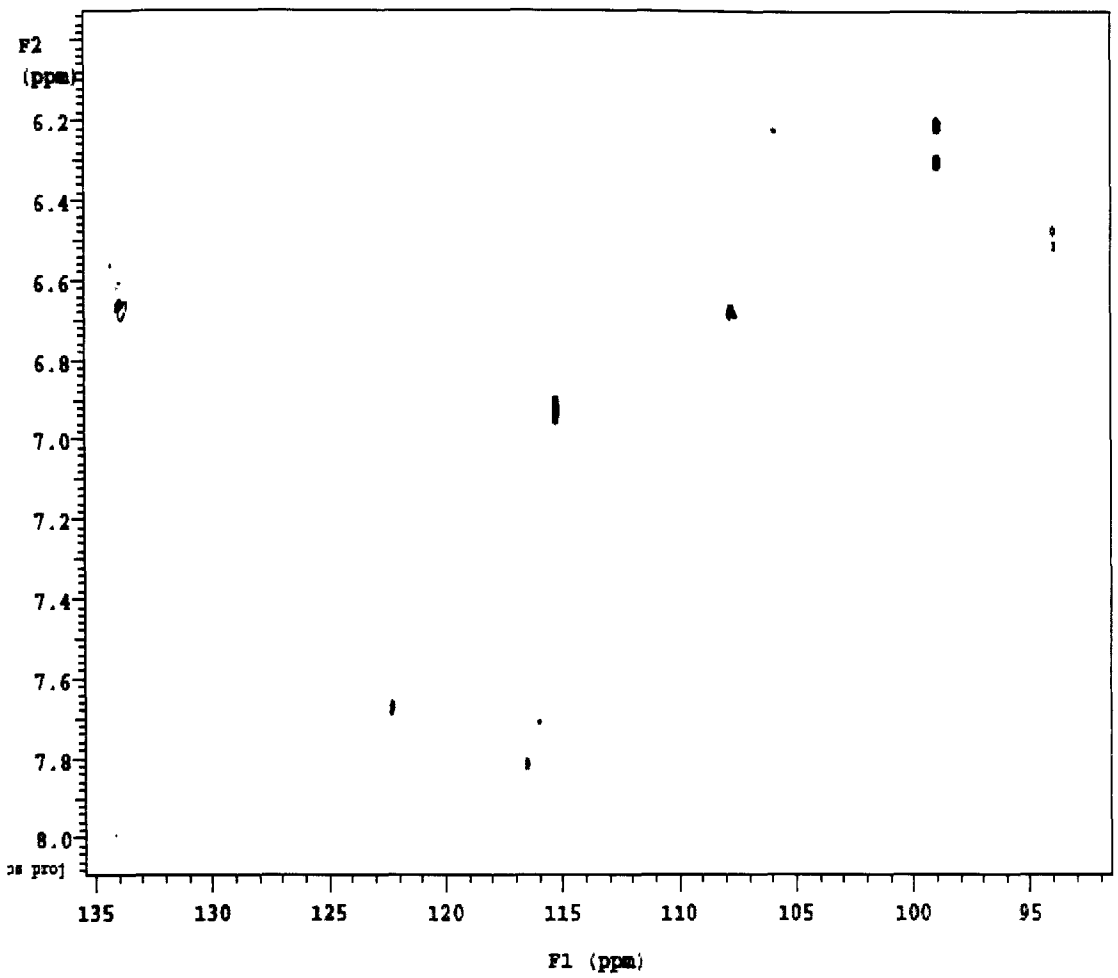

Figure F-11 Upfield gHSQC NMR of DPG and blueberric acid isolated from fraction 28,8.

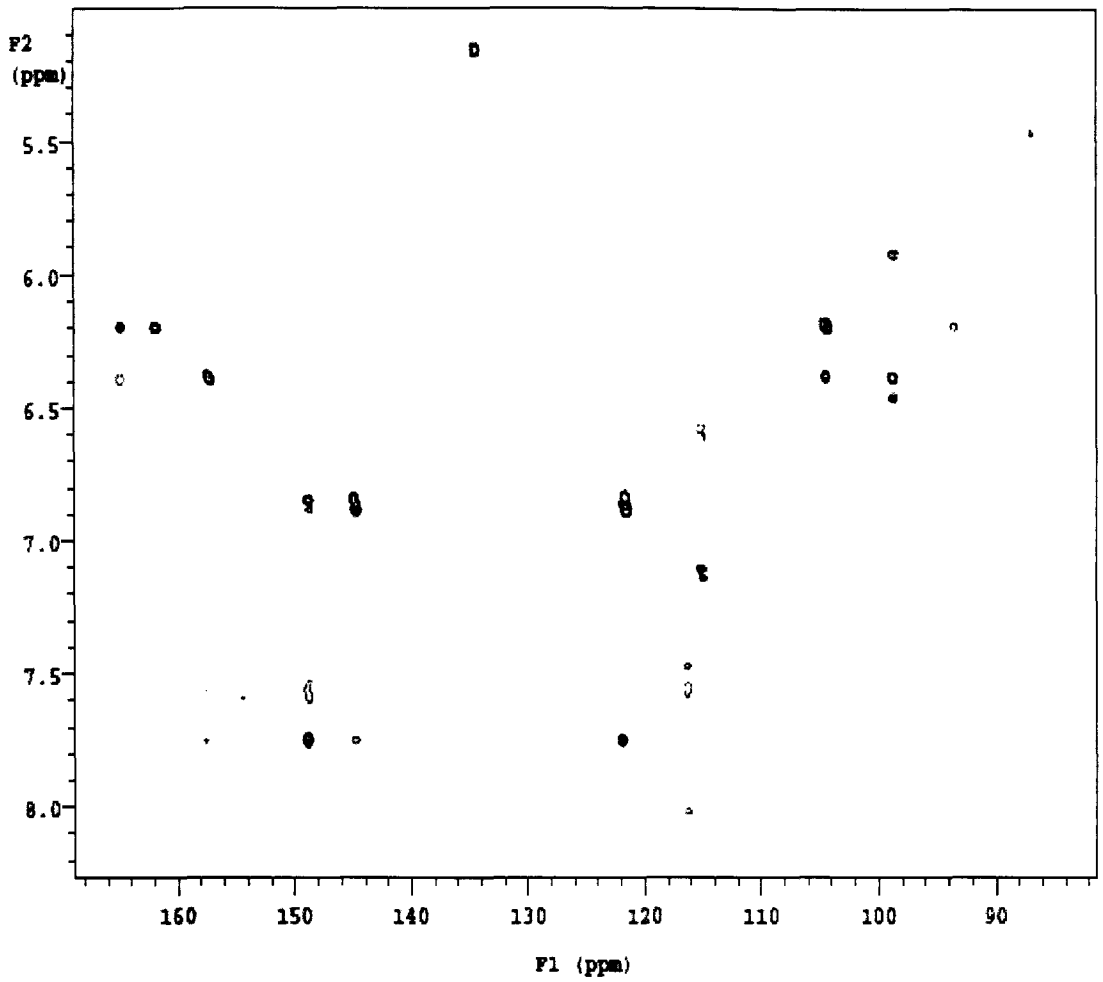

Figure F-12 Downfield proton / downfield carbon of gHMBC NMR of DPG and blueberric acid isolated from fraction 28,8.

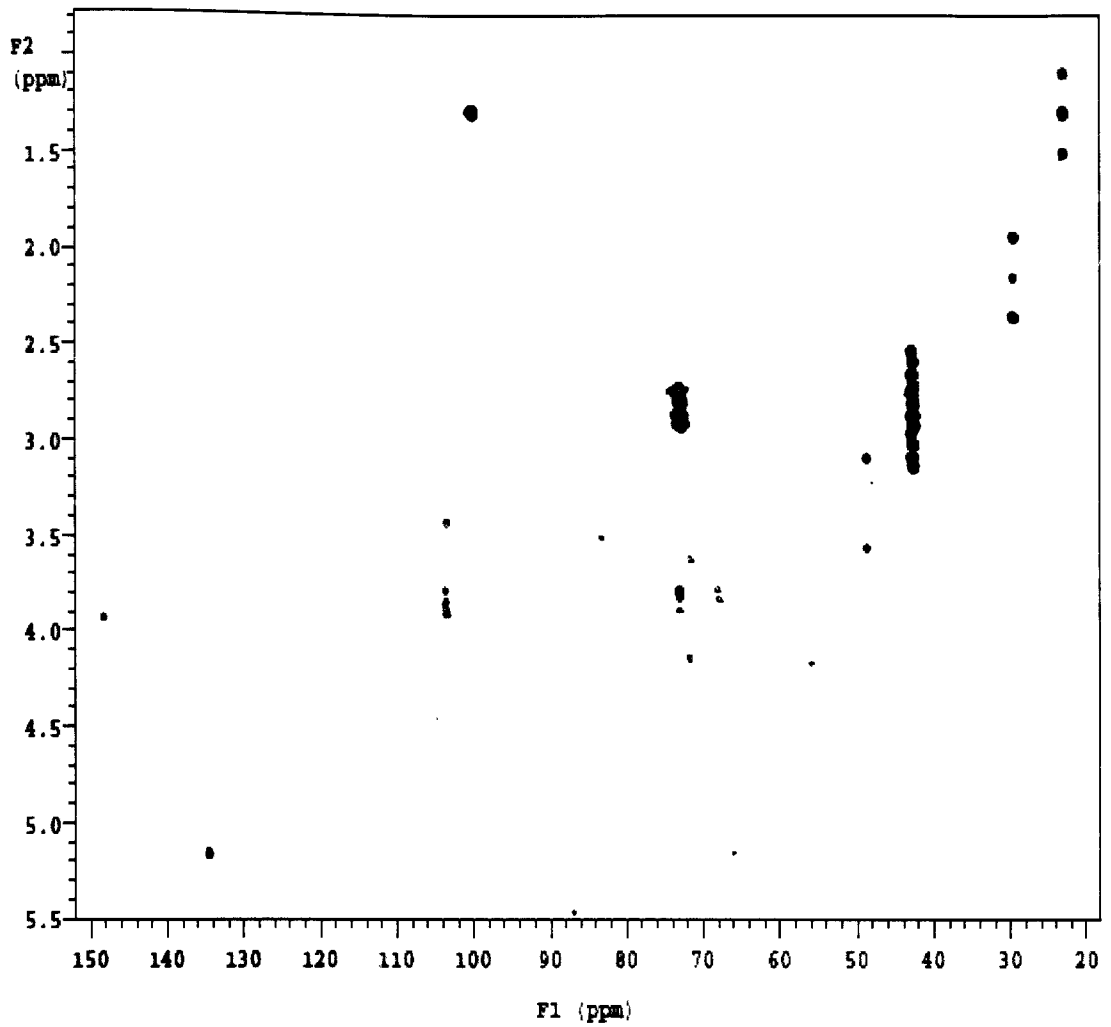

Figure F-13 Upfield proton / downfield carbon gHMBC NMR of DPG and blueberric acid isolated from fraction 28,8.