Molecular Mechanisms of Neuroprotection by an Alternative Drug *Scutellaria lateriflora*

by

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A dissertation submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Doctor of Philosophy

Auburn, Alabama
August 02, 2014

Key words: American skullcap, Anxiety, Memory, Neuroprotection, *Scutellaria lateriflora*

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Abstract

Anxiety is one of the most prevalent neuropsychological disorders around the world. In the United States of America (USA), it is a serious health problem affecting a large number of people and is considered as one of the common disorder seen in the primary health care. Based on the current understanding of the pathologic mechanisms of anxiety, there are limited pharmacological and non-pharmacological therapies. Benzodiazepine (the current first line of therapy) has severe adverse effects such as anterograde amnesia, tolerance, psychomotor impairment, memory disruption, impaired psychomotor function, paradoxical anxiety or aggression, risks of accidents and even mortality. A double blind, placebo-controlled clinical trial indicated that commercial preparations *of Scutellaria lateriflora* have therapeutic benefits in anxiety with no evidence of toxicity. The purpose of this study was to examine the biological activities of *S. lateriflora* that may contribute to its neuroprotective mechanisms. The research study was conducted in four phases. The first two experimental approaches determined anti-oxidative potential and protective activities of *S. lateriflora* against oxidative stress induced neuronal cell death. The third experimental approach was designed to examine anti-inflammatory and immunomodulatory activities of *S. lateriflora*. Finally, we examined the effects of *Scutellaria lateriflora* on synaptic plasticity in the form of long-term potentiation (LTP). *S. lateriflora* exhibited anti-oxidative, anti-inflammatory and immunomodulatory activities, however, it suppressed synaptic plasticity. These results indicate neuroprotective activities of *S. lateriflora* against oxidative stress induced cell death and have anti-inflammatory activities.

Acknowledgments

I praise and thank the Almighty, the most merciful for blessing me and giving me the strength and support, which helped me to finish this work. I would like to dedicate my work to my parents, my family and friends. It's with their encouragement, love and inspiration, I have been able to quench my desire for higher studies and strive for excellence in research.

I would like to express my deep appreciation and sincere thanks to Dr. Barbara W Kemppainen for her continuous guidance, encouragement, support and patience throughout my doctoral studies at Auburn. I express my sincere gratitude to my committee members Dr. Dean D Schwartz, Dr. Kellye Joiner, Dr. Muralikrishnan Dhanasekaran and Dr. Frederik W Van Ginkel for keen supervision and contributions of resources, time and technical assistance, making this project possible. I would also like to thank Dr. Manal A Buabeid for helping me with electrophysiology techniques. I highly appreciate Dr. Vishnu Suppiramaniam and Dr. Raj Amin for generously letting me use his laboratory resources. I would like to express my special thanks to Dr. Chris Newland, for serving in my graduate committee as an official outside reviewer of this dissertation. My thanks also go to my colleagues, Dr. Daniel Katz, Dr. Wanshu Ma, Dr. Gayani Nannayakara, Ms. Mengyuan Zheng, Ms. India Napier, Dr. Mrigendra Rajput and Dr. Ahmad, Mr. Dwipayan Bhattacharya and Mr. Shobhrajeet Bhattacharya for their support and good-spirited discussions during this study. I would especially like to thank Dr. Manuj Ahuja my colleague and friend for his assistance in various avenues of this work. I would like to extend my appreciation and gratitude to all the faculty members, staff and fellow students at Dept. of Anatomy, Physiology and Pharmacology and Pharmacal Sciences, Auburn University. I would like to

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List of Abbreviation

15-LOX	15 Lipoxygenases
5HT7R	Serotonin-7 Receptor
8OHdG	8-hydroxydeoxyguanosine
8-OXO-dG	8-oxydeoxyguanosine
AC-DEVD	Acetyl Asp-Glu-Val-Asp 7-Amido-4-Methyl Coumarin (AMC)
AD	Alzheimer Disease
ALS	Amyotrophic Lateral Sclerosis
AMPAR	Alpha Amino-3-Hydroxy-5-Methylisoxazole-4-Propionic Acid Receptors
BBB	Blood Brain Barrier
BDNF	Brain-Derived Neurotrophic Factor
BDZs	Benzodiazepines
BSA	Bovine Serum Albumin
CA1	Cornus Ammonis 1 (A region of hippocampus circuit which yield a high output)
cAMP	cyclic Adenosine Monophosphate
CAT	Catalase
CD14	Cluster of Differentiation
CFSE	Carboxyfluorescein Succinimidyl Ester
CNS	Central Nervous System
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
$cPLA_2$	Phospholipase A
CREB	Cyclic Adenosine Monophosphate (cAMP) response element binding
CRP	C-reactive protein
DCF-DA	DiChlorodihydroFluorescein Diacetate

DCFH-DA	2'-7'-DichloroDihyDroFluorescein Diacetate
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Di-Methyl-sulfoxide
DPPH	1,1-diphenyl-2-picrylhydrazyl radicals
FACS	Fluorescence Activated Cell Sorting
FBS	Fetal Bovine Serum
fEPSP	field Excitatory Post Synaptic Potentials
FITC	Fluoresceinisothiocyanate
GABA	Gamma-Aminobutyric Acid
GABAR	Gamma Amino Butyric Acid Receptors generation or enzyme activities
GLO1	Glyoxalase
GMCSF	Granulocyte Monocyte Colony Stimulating Factor
GPX	Glutathione Peroxidase
GSAD	Generalized Social Anxiety Disorder
GSH	Glutathione
GSR	Glutathione Reductase
GSR1	Glutathione Reductase
GSSG	Glutathione Disulfide
H19-7	Hippocampal Cells
HADS	Hospital Anxiety and Depression Scale
HD	Huntington disease
IC50 value	Concentration required of a chemical to inhibit 50% of reactive oxygen species
IFN-γ	Interferon gamma
IGF-1	Insulin like growth factor-1
IL-1	Interleukin-1
IL-12	Interleukin 12
IL-6	Interleukin-6
iNOS	Inducible Nitric Oxide Synthase
LOX	Lipoxygenases

LPS	Lipopolysaccharides
LTP	Long Term Potentiation
MCAO	Middle Cerebral Artery Occlusion
MHC	Major Histocompatibility Complex
mKRB	Modified Krebs-Henseleit Buffer
MoDCs	Monocyte-Derived Dendritic Cells
MTT	(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NF-kB	Nuclear Factor K-Light-Chain-Enhancer of Activated B cells
NMDAR	N-Methyl-D-Aspartate Receptors
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
NR2A	N-Methyl-D-Aspartate Receptor Subunit
NR2B	N-Methyl-D-Aspartate Receptor Subunit
NSAIDs	Non-Steroidal Anti-Inflammatory Drugs
OCD	Obsessive Compulsive Disorder
PBMCs	Peripheral Blood Mononuclear Cells
PC12	Pheochromocytoma Cells
pCREB	Phosphorylated cAMP Response Element-Binding Protein
PD	Parkinson Disease
PIC	Pro-Inflammatory Cytokines
P_o	Open Probability of a Channel
PrP	Prion Protein
PTSD	Post Traumatic Stress Disorder
PVDF	Poly Vinylidene Di Fluoride
RAW 264.7	Macrophage cells
ROS	Reactive Oxygen Species
RPMI	Roswell Park Memorial Institute medium
SLE	Alcoholic Extract of *Scutellaria lateriflora*

SNRIs	Serotonin-Norepinephrine Reuptake Inhibitors
SOD	Super Oxide Dismutase
SSRIs	Selective Serotonin Reuptake Inhibitors
TBA	ThioBarbituric Acid
TBS	Theta-Burst Stimulation
TBST	Tris-Buffered Saline containing 0.1% Tween-20
TCA	TriChloroacetic Acid
TGF-β1	Transforming growth factor beta
TMPD	N,N,N, N tetramethyl-p-phenylenediamine
TNF-α	Tumor Necrosis Factor-α
TSE	Transmissible Spongiform Encephalopathies
XAO	Xanthine Oxidase
τ1	Open or close time of a channel in bimodal histogram
τ2	Open or close time of a channel in bimodal histogram

1. INTRODUCTION

The occurrence of anxiety disorders is high, with a lifetime prevalence rate of approximately 25% in the US population (Kessler et al., 2005). Anxiety behavior is a response to exaggerated apprehension and does not correspond to the actual situation (Ohl et al., 2008). Insomnia, headache, backache, sweating, trembling and frequent urination are a few common symptoms of pathological anxiety. Based on symptoms, severity and etiology, pathological anxiety can be divided into various classes and contributes to pathogenesis of a range of other neuropsychiatric conditions (Lavie and Milani, 2004).

Not only humans, but also animals are affected by anxiety and anxiety disorders. Among neuropsychological disorders, separation anxiety is the most common behavior disorder in pets (Overall, 1997; Sherman, 2008). Vocalization and inappropriate defecation and urination are common behavioral manifestation exhibited by dogs suffering from separation anxiety. Some pets also show other signs, including hyper-salivation, diarrhea, circling, digging, excessive licking (McCrave, 1991). However, as in humans, the etiology of the anxiety disorder in animals is uncertain, but could be caused by inheritable characteristics and environmental factors (Serpell and Jagoe, 1995).

Treatment of anxiety disorders in humans and animal patients is important for a number of reasons. In humans, anxiety can cause significant fear, nervousness, apprehension and have a serious impact on daily life. Additionally, depression is often associated with anxiety disorders. Up to 70% of the patients that are suffering from some kind of anxiety disorders also suffer from at least one major depressive episode (Stein et al., 1990; Van Ameringen et al., 1991). Anxiety in animals can become so intolerable to the owner, that without treatment, it may lead to abandonment or even euthanasia of the pet. (Houpt et al., 1996; Miller et al., 1996).

Benzodiazepines are a commonly used anxiolytic drug therapy for anxiety disorders (Sadock et al., 2000); however, long term use of benzodiazepine causes a range of adverse effects including loss of memory and addiction (Woods et al., 1987). Another group of anxiolytic drugs, e.g. selective serotonin re-uptake inhibitors and serotonin-norepinephrine re-uptake inhibitors, are ineffective and exhibit a lack of response in many patients (Katzman, 2009). Approximately 43% of human patient suffering from anxiety disorders use some form of complementary therapy (Eisenberg et al., 1998). Among alternative therapy, the use of herbal medicine is the most popular treatment for neuropsychological disorders (Astin, 1998; Wong et al., 1998) For example, St John's wort and ginkgo have been used to treat depression and dementia, respectively. Similarly, *S. lateriflora*, also known as American skullcap, is a native plant of North America and has been used by Americans and Europeans as a nerve tonic for more than 200 years. Clinical trials report that *S. lateriflora* has anxiolytic activities with little evidence of toxicity (Wolfson and Hoffmann, 2003). Anxiolytic activities of *S. lateriflora* is attributed to the presence of flavonoids and amino acids that constitutes approximately 25% of dry weights of alcoholic extracts (Bergeron et al., 2005).

The purpose of this study was to examine the biological activities and mechanism of action of *S. lateriflora* extracts contributing to its anxiolytic activity. The research study was conducted in four phases. The first two phases determined whether *S. lateriflora* showed anti-oxidative activities and prevented oxidative stress induced neuronal cell death. The third phase was designed to examine anti-inflammatory and immunomodulatory activities of *S. lateriflora*. Finally, the fourth phase evaluated effects of *S. lateriflora* on synaptic plasticity in the form of long-term potentiation (LTP). *S. lateriflora* exhibited anti-oxidative, anti-inflammatory and immunomodulatory activities; however, it decreased synaptic plasticity. These results indicate neuroprotective activities of *S. lateriflora* that can explain, in part, the anxiolytic effects.

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2. LITERATURE REVIEW

2.1. Introduction

Anxiety is one of the most prevalent neuropsychological disorders around the world. In the United States of America (USA), it is a serious health problem affecting a large number of people and is considered one of the common disorders seen in primary health care. Approximately, eighteen percent of the adult population is affected by anxiety in the USA (Kessler et al., 2005). Anxiety is a broad and common behavior expression for numerous ailments that induce significant fear, nervousness, apprehension, and worry. Both anxiety and fear can result from certain real or imagined dangers, but the former is usually a response to an unknown threat, while the latter is a response to a definitive threat. Anxiety deals with complex, negative emotions. Mild anxiety is vague and unsettling, while severe anxiety can be extremely debilitating, having a serious impact on daily life. However, the neuropsychological behavioral changes seen in the different forms of anxiety are significantly distinct from depression, psychosis or bipolar disorder. Psychosis is a serious mental disorder in which a patient sees and hears things that are not present. In bipolar disorder, a patient experiences shifts in mood from depression to euphoria. Depression is an overwhelmingly sad feeling which can interfere with everyday life if it occurs for longer periods of time. Unlike a mild form of anxiety, severe anxiety can lead to various anxiety disorders, including obsessive compulsive disorder. Based on severity, etiology and nature of the disease (e.g. acute versus chronic), anxiety disorders can be categorized into eight classes.

2.1.A. Different types of anxiety

There are several types of pathological anxiety and can be divided into : 1.) Generalized Anxiety Disorder (GAD), 2.) Panic Disorder, 3.) Social Phobia, 4.) Simple Phobia, 5.) Social

Anxiety Disorder, 6.) Post-Traumatic Stress Disorder (PTSD), 7.) Obsessive Compulsive

Disorder (OCD) and 8.) Separation Anxiety Disorder (Table 2.1).

 (www.nimh.nih.gov, www.nami.org)

Table 2.1. Classes of anxiety disorders and factors involved in their mechanism of action.

Anxiety Disorders	Factors Involved in Mechanisms of Anxiety
• Generalized anxiety disorder • Panic disorder • Social phobia • Simple phobia • Social anxiety disorder • Post-traumatic stress disorder • Obsessive compulsive disorder • Separation anxiety disorder	• Genetic involvement • Hyperarousal • Gamma-aminobutyric acid • Serotonin • Change in stress hormone levels • Oxidative stress • Inflammation • Immunity

Severe anxiety is a condition in which the patient's behavior, daily routine and ability

to sleep are severely impaired. Generalized Anxiety Disorder is a chronic, non-specific

condition, spurred on by the stress from daily life events, usually lasting for a longer period

of time (6 months). Anxiety occurs more often in females as compared to males. Panic

disorder arises abruptly and peaks due to an inappropriate, over-stimulus of the sympathetic

nervous system. Social phobia is the overwhelming fear of everyday social situations. Simple

phobia is a strong, irrational fear to a specific object or situation (http://www.nami.org).

Obsessive compulsive disorder is a combination of distressing, repetitive thoughts followed

by repetitive behavior to relive anxiety. Post-traumatic stress disorder is a result of a

traumatic life event. It is a form of anxiety which is an outcome of earlier trauma due to war,

rape, hostage situations, or an accident (http://www.nami.org). Post-traumatic stress disorder

often leads to flashbacks and behavioral changes intended to avoid certain stimuli

(www.nami.org). Thus, anxiety can reduce the quality of life and adversely affect occupational and social activities. Therefore, there is a need to further examine the exact pathophysiology of anxiety disorders

2.1.B. Mechanisms of anxiety

There are several mechanisms involved in the etiopathology of anxiety such as 1.) genetic involvement, 2.) hyperarousal, 3.) alterations in the gamma-aminobutyric acid (GABAergic) or 4.) serotonergic neurotransmission, 5.) change in stress hormone levels and 6.) oxidative stress. It has been suggested that genetic and biological disposition play important roles in the pathogenesis of this disease (Brawman-Mintzer and Lydiard, 1996; Villafuerte and Burmeister, 2003). Hyperarousal is a pathological condition associated with anxiety, insomnia and sometimes seizures. Hyperarousal is a form of anxiety in which patients exhibit increased psychological and physiological tension marked by such effects as reduced pain tolerance, anxiety, exaggerated startle responses, insomnia, fatigue, and accentuation of personality traits (Tillman et al., 2012). Abnormalities in the γ-aminobutyric acid–mediated (GABAergic) and serotonin-mediated (serotonergic) neurotransmission may also be involved in the etiology of anxiety disorders (Brawman-Mintzer and Lydiard, 1996). There is a decreased function of serotonin and GABA in patients with anxiety, usually associated with increased fear and sympathetic arousal due to hyperactivity of the thalamus, hypothalamus, cortex and amygdala (Brawman-Mintzer and Lydiard, 1996, Bishop et al., 2007). Panic attacks are associated with increased heart rate, shortness of breath, chest pain, and elevated stress, reflecting a surge in sympathetic activity. Decreased serotonergic activity leads to the loss of a "feel good" sensation and increased overall brain hyperactivity (due to a lack of inhibition).Sometimes, when a person is anxious, there may be no direct external stressor to account for the body's stress response, although stress is the main reason for anxiety. The amygdala is an important part of the brain which processes emotions and

memory, and in an anxious person stimulates increased norepinephrine production. The hypothalamus and pituitary increase corticotropin-releasing hormone (CRH) and adrenocorticotropic hormone (ACTH) production. It has been reported that reduced anxiety behavior is associated with reduction of functional CRH hormone (Peter et al., 1998).

There is a close correlation between oxidative stress and anxiety in patients suffering from obsessive–compulsive disorder, panic disorder, and is considered a high trait in anxiety (Bouayed et al., 2009). In fact, oxidative stress in the brain is potentially involved with pathogenesis and a risk factor for behavioral anomalies and psychological disorders (Ng et al., 2008). Additionally, inflammation and immunity also play roles in the pathogenesis of neuropsychological disorders, including anxiety. A range of factors contribute to the pathogenesis of anxiety; however our discussion is focused on roles of oxidative stress, inflammation and immunity in anxiety disorders, as these factors play important roles in a variety of other neuropsychological disorders.

2.2. Anxiety disorders: Role of Inflammation, Oxidative Stress and Immunity

Inflammation and oxidative stress in brain tissue, and the systemic immune response of the body play important roles in the complex pathogenesis of anxiety disorders. Substantial clinical and etiological differences exist among different anxiety disorder subtypes, contributing to the complexity and heterogeneity of anxiety disorders. The translational differences between animal models of anxiety behaviors and human anxiety symptoms further contribute to the incomplete understanding of the pathogenesis of anxiety disorders.

2.2. A. Role of Inflammation in the Pathogenesis of Anxiety

Systemic inflammation plays a key role in various neurodegenerative and neuropsychological diseases, including anxiety disorders. The blood–brain barrier (BBB), a selective permeability barrier, is formed by capillary endothelial cells that separate some components of circulating blood from the brain extracellular fluid (BECF) in the central

nervous system (CNS). Pro-inflammatory cytokines (PICs) easily cross the BBB from the systemic circulation (Banks and Erickson, 2010; Maes, 2011; Quan and Banks, 2007). Additionally, these PICs cause reduced tightness of capillary endothelial cell junctions, resulting in facilitating PICs entry into the brain. These PICs instigate localized inflammation in the central nervous system through the activation of microglial cells (Qin et al., 2007). Microglia cells are macrophage cells found in the CNS which produce inflammatory mediators after PICs-induced activation (Wager-Smith and Markou, 2011).
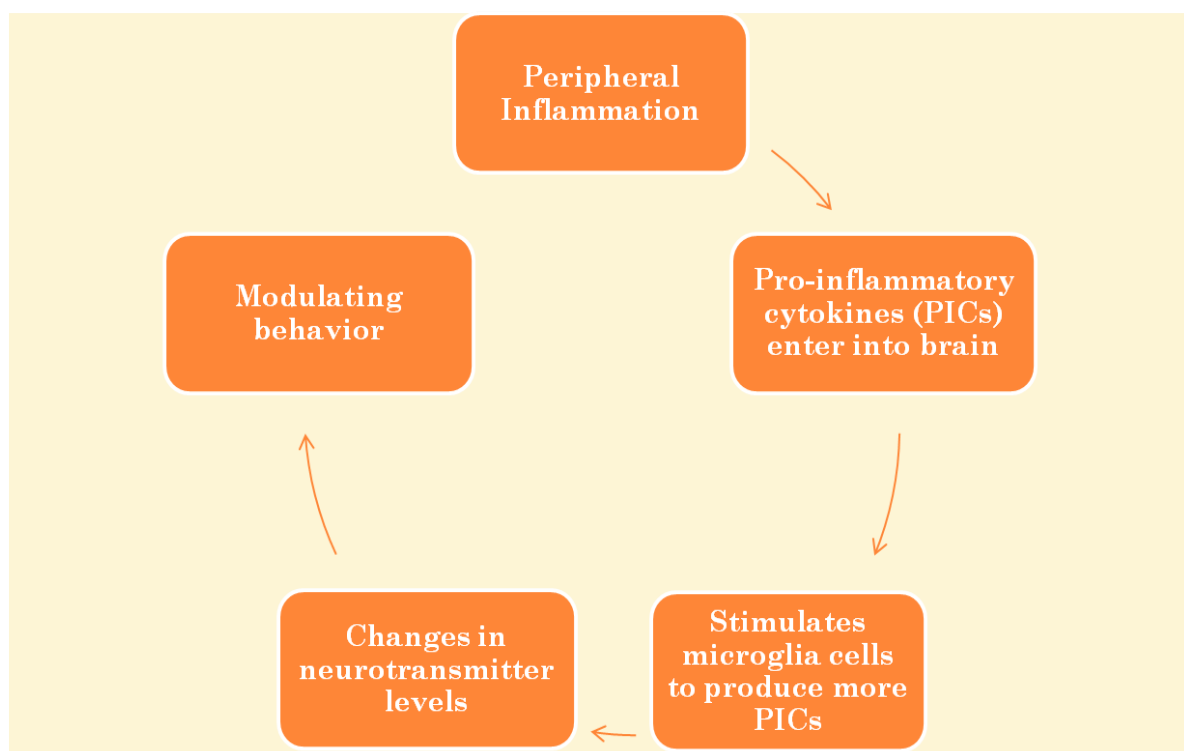
Figure 2.1. Inflammation induces anxiety behavior by modulating levels of neurotransmitters.

Additionally, systemic inflammation may affect brain functions by modulating brain signals through the afferent fibers of vagus nerves and by "the neuronal axes" carrying "viscero-sensorily" signals (e.g., inflammation) to specific brain areas, which are involved in depression and anxiety. These signals are converted into information about the internal body state to different parts of brain (e.g. caudal medulla, hypothalamus and brainstem areas), by changing levels of neurotransmission in the brain and thus modulating behaviors, such as

arousal, anxiety and depression (Gaykema et al., 2009). Pro-inflammatory cytokines also affect enzyme activity in neurons, affecting in the metabolism of neurotransmitters and thereby modulating their concentrations. For example, PICs induce expression of indoleamine 2, 3-dioxygenase, an enzyme responsible for the degradation of serotonin and tryptophan into kynurenine and quinolinic acid, respectively. Kynurenine and quinolinic acid are known to induce anxiety, depression and oxidative stress in neurons by damaging normal mitochondrial respiratory mechanisms (Leonard and Maes, 2012; Moylan et al., 2013) (Figure 2.1).

Interleukin-6 (IL-6) is an important pro-inflammatory cytokine produced by innate and adaptive immune cells. C-reactive protein (CRP) is a protein found in the blood, the levels of which rise in response to IL-1 and IL-6. Researchers have found a positive correlation between pro-inflammatory molecules in systemic blood circulation and anxiety disorders in human patients. Clinically anxious individuals, as determined by a hospital anxiety and depression scale (HADS), demonstrated significantly higher levels of IL-6 and lower levels of serum cortisol, but showed no difference in CRP, compared with non-anxious individuals (O'Donovan et al., 2010). The HADS is commonly used by physicians to determine the extent of anxiety and depression that a patient is experiencing. Abnormal psychophysiological responses may range from behavior manifestations to psychological stress in anxiety disorders. Heart rate, respiration rate, electrodermal activity, and back muscle tension are a few examples of psychophysiological responses. Studies suggest that psychological stress manifested by abnormal psychophysiological responses are related to increased production of PIC such as, tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6) and interferon-gamma (IFN-γ), coupled with decreased production of anti-inflammatory cytokines interleukin-10 (IL-10) and interleukin-4 (IL-4) (Maes, 2011; Maes et al., 2011b). Results of others studies suggested that increased psychological stress is associated with

lower levels of TNF-α (Chandrashekara et al., 2007). Similarly, the relationship between other anxiety disorders and PICs varies across research studies. Some studies found decreased levels of plasma TNF-α, IL-1β and IL-6 in OCD patients. However, other studies determined increased levels of these plasma pro-inflammatory cytokines in OCD patients (Brambilla et al., 1997; Denys et al., 2004; Konuk et al., 2007; Monteleone et al., 1998). Differences in the type of stress, study population and psychological stress measurement techniques might explain inconsistent results. Studies comparing lipopolysaccharide (LPS) induced production of IL-6 in obsessive compulsive disorders and Generalized Social Anxiety Disorder (GSAD) patients, found decreased level of IL-6 in OCD patients but no change in GSAD patients (Fluitman et al., 2010).

Based on the assumption that inflammation contributes to anxiety disorders, researchers used cytokine based immunotherapy to treat anxiety patients. Interferon-α based immunotherapy resulted in more severe depression and anxiety. Patients receiving treatment with interferon therapy demonstrate increased anxiety symptoms (Maes et al., 2001). A number of mechanisms have been proposed to explain an inflammatory mediated pathogenesis for mood disorders, including inter-related influences on neurogenesis and neuronal survival, epigenetics, regulation of monoamine neurotransmitters and regulation of cell-mediated immunity (Maes, 2011). Research studies indicate that peripheral inflammation may modulate brain neurotransmission independent of the inflammatory state of the CNS (Banks and Erickson, 2010; Maes et al., 2011a; Maes et al., 2011b; Quan and Banks, 2007). However, whether peripheral inflammation- induced changes in neurotransmitters in the brain can be observed without affecting anxiety remains to be determined. In conclusion, results of most research studies indicate roles of inflammation in anxiety disorders. Similar to inflammation, oxidative stress is also associated with a range of neuropsychological disorders.
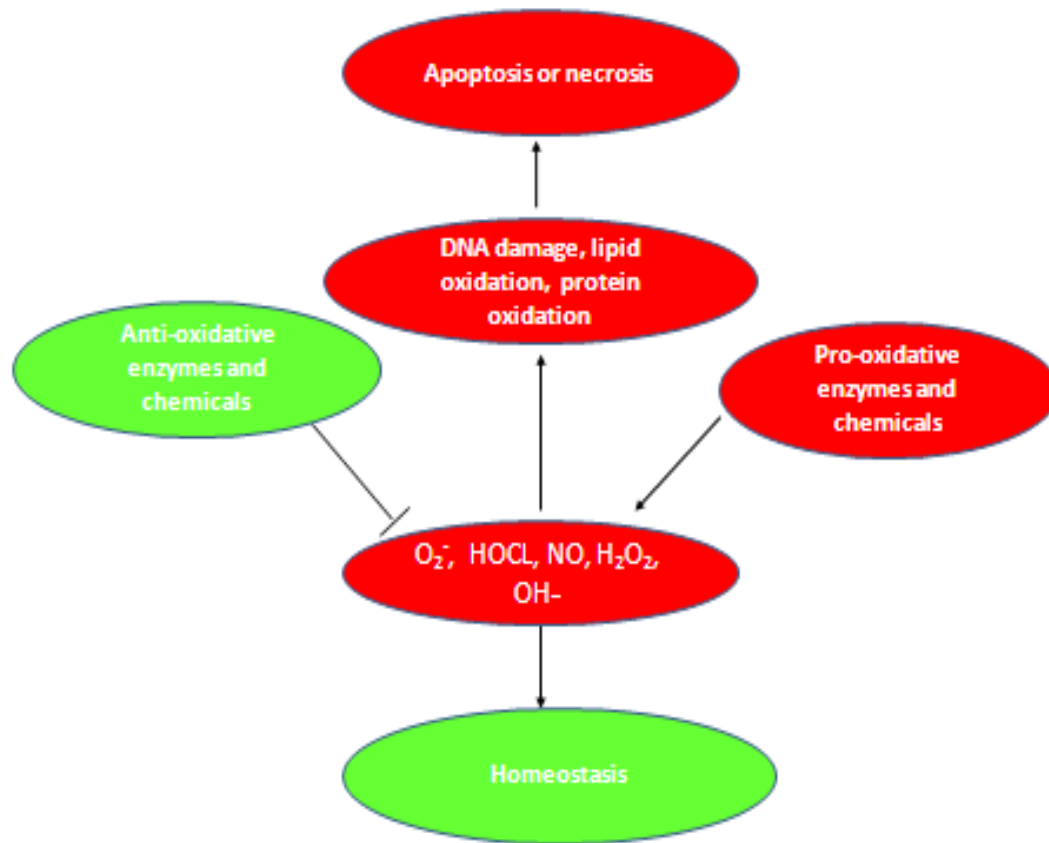
Figure 2.2. Oxidative stress can lead to neuronal cell death via disruption of cellular macromolecules.

2.2. B. Role of Oxidative Stress in Pathogenesis of Anxiety

Secondary products of oxidative phosphorylation at low to moderate concentrations are required in normal cellular processes such as mitosis and apoptosis (Valko et al., 2007). However, increased levels of reactive oxygen species and reactive nitrogen species beyond the normal antioxidant capacity of cells, can lead to alterations in the structure and function of membrane fatty acids and proteins, and can modulate or damage DNA and mitochondrial function leading to cell death via necrosis or apoptosis (Figure 2.2) (Maes et al., 2011a). Markers of oxidative stress are frequently reported in animal models of anxiety and human subjects suffering from anxiety disorders (Hovatta et al., 2010). Increased lipid peroxidation has been coupled to the severity of anxiety disorders including OCD, PTSD and social phobia

in human patients (Atmaca et al., 2008; Chakraborty et al., 2009; Ersan et al., 2006; Ozdemir et al., 2009; Tezcan et al., 2003). Furthermore, to compensate for the excessive generation of reactive oxygen species in oxidative stress, enhanced activity of the antioxidant enzymes glutathione reductase (GSR), catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPX) have been reported in human patients suffering from anxiety disorders (Atmaca et al., 2008; Ozdemir et al., 2009; Tezcan et al., 2003). Glutathione reductase, is an enzyme that reduces glutathione disulfide (GSSG) to the sulfhydryl form glutathione (GSH) , an important cellular antioxidant (Meister, 1988). Superoxide dismutase converts superoxide oxygen molecules to hydrogen peroxide and CAT which convert hydrogen peroxide to non-toxic molecular oxygen. Lipid hydroperoxides are neutralized by GPX enzymes that convert lipid radicals to their corresponding alcohols (Figure 2.2).

Oxidative stress appears to be associated with psychological stress and specific brain-regions develop cellular damage characteristic of oxidative stress (Hovatta et al., 2010). It has been reported that chronic, mild psychological stress is associated with increased superoxide production in the mitochondria of rat hippocampus, prefrontal cortex and cortex (Lucca et al., 2009). Similarly, the occurrence of stress corresponds to lipid peroxidation, oxidative damage to DNA, and reduced antioxidant activity in plasma (Sivonova et al., 2004). Interestingly, increased depression levels and the inability to cope with depression are associated with elevated 8-hydroxydeoxyguanosine (8-OHdG) levels (Irie et al., 2005). One of the major products of DNA oxidation is 8-oxo-dG (8-oxydeoxyguanosine) and increased concentrations of 8-oxo-dG are a marker of oxidative stress in the cell (de Souza-Pinto et al., 2001). A report suggests that certain antioxidant proteins, also act as neurotrophic factors, are secreted by neurons are essential for neuronal survival and are known to prevent the affected neurons from undergoing apoptosis. These neurotrophic factors e.g nerve growth factor, brain derived neurotrophic factor (BDNF), also stimulate differentiation of progenitor cell, to form neurons

and regulate neurogenesis and neuroplasticity (Lee and Son, 2009). Among the neurotropic factors, BDNF is a potential anti-oxidant chemical in the brain (Chan et al., 2010). Interestingly, muscle is a major source of BDNF, and the expression of BDNF is increased after physical activity. Reports link BDNF to improved spatial learning and memory (Aguiar Jr et al., 2011; Rasmussen et al., 2009).

Data from animal studies suggests that BDNF plays a role in anxiety. An intra-hippocampal injection of BDNF in rats led to an increase in anxiety, assessed by facilitatory avoidance and the light–dark test (Casarotto et al., 2012). Social deprivation stress leads to the development of anxiety in mice, and evidence suggests this is modulated by reductions in BDNF (Berry et al., 2012). In a cross sectional study of humans, plasma BDNF levels were negatively associated with the severity of obsessive-compulsive disorder, interpersonal sensitivity and anxiety (Bhang et al., 2012). BDNF has been suggested as a modulatory factor in the development of PTSD (Rakofsky et al., 2012). Recent evidence suggests that peripheral BDNF levels correlate with central (hippocampal) BDNF levels in a variety of species, e.g. rat, mouse, pig (Klein et al., 2011; Sen et al., 2008). However, the exact mechanisms mediating BDNF transport across the BBB are still under investigation. (Sen et al., 2008). Indirectly, BDNF may influence the brain by effecting peripheral tissues, resulting in the release of other factors.

In addition to direct association of inflammation and oxidative stress with anxiety disorders, interactions between inflammation and oxidative stress also influence the pathogenesis of pathological anxiety.

2.2.C. Pathogenesis of Anxiety Involving Interactions of Oxidative Stress and Inflammation

Sub-chronic oxidative stress induces down-regulation of BDNF, glyoxalase 1 (GLO), and glutathione reductase (GSR) in nerve cells. Similar to GSR, GLO neutralizes reactive

aldehydes produced as a normal part of cellular metabolism (Vander Jagt, 1989). Sub-chronic oxidative stress down-regulates GLO and GSR via induction of calpain expression in the hippocampus, predisposing the hippocampus to a state of increased protein glycation and subsequent further oxidative stress (Salim et al., 2011). Sub-chronic oxidative stress was induced in male Sprague–Dawley rats by injecting l-Buthionine-sulfoximine (BSO) (300 mg/kg body weight i.p.) daily for 7 days. Calpain is a calcium-dependent proteolytic enzyme that is expressed in mammalian cells. Its activation has been linked to oxidative stress. Oxidative stress, and calpain activation (Shumway et al., 1999), induces NFκB (nuclear factor kappa-light-chain-enhancer of activated B cells) transcription. Transcription factor NF-kB is a protein complex ubiquitously expressed in almost all mammalian cell types. It is involved in cellular responses to a range of stimuli, including oxidative stress, and leads to the production of IL-1, TNF-α and other inflammatory mediators (Gilmore, 2006; Salim et al., 2011). Augmented inflammatory responses can further induce an increase in oxidative stress by stimulating pro-oxidant enzymes and/or inhibition of antioxidant enzymes. Increased oxidative stress can inhibit BDNF expression and its function by leading to cellular damage and the inhibition of cellular processes, which cause neuroplasticity. Neuroplasticity refers to changes in the structure of nerve cells that pass an electrical or chemical signal to another cell (Pascual et al., 2011). Inflammation and oxidative stress are both associated with the pathogenesis of a range of neuropsychological disorders. Increased PIC molecules cause oxidative stress and vice versa. Almost all major PICs can induce oxidative stress via inducing pro-oxidant enzyme activities. IFN- γ increases nitrous oxide synthesis by increasing inducible nitric oxide synthase (iNOS) gene expression (Maes et al., 2012). Similarly, IL-1β and TNF-α increase reactive oxygen species generation by stimulating arachdonic acid production via activation of Nicotinamide Adenine Dinucleotide Phosphate (NADPH) oxidase (Chenevier-Gobeaux et al., 2007). NADPH oxidase, an pro-oxidative

enzyme present in the plasma membrane of cells, generates superoxide by transferring electrons from NADPH and coupling these electrons to oxygen to produce superoxide anion, a reactive free-radical.

The molecular mechanisms by which PICs induce oxidative stress in neurons have been studied. Binding of PICs and activation of cell surface receptors, results in modulation of function of NF-kB and the cyclic Adenosine Monophosphate (cAMP) response element binding (CREB) transcription factors. These activated transcription factors NF-kB, CREB regulate the activities of nitric oxide synthase (NOS), cyclo-oxygenase 2 (COX2) and NADPH oxidase (Hovatta et al., 2010), leading to modulation of reactive oxygen and nitrogen species generation in the cell. Increased activation of nuclear CREB transcription factor has been associated with reduced anxiety and increased viability of neurons (Barrot et al., 2005; Mantamadiotis et al., 2002). Suppression of activities of these pro-oxidant enzymes reduces anxiety behaviors. For example, increasing serotonin receptors resulted in reduced activity of NOS and led to an anxiolytic effect. The anxiolytic affects was mediated by an enhanced phosphorylation of CREB transcription factor resulting from suppressed NO production (Zhang et al., 2010). Reduced NADPH oxidase activity suppresses anxiety behavior resulting from induced oxidative stress. Increased NADPH oxidase activity, in hippocampal regions of rat brain has been associated with enhanced anxiety behaviour (Masood et al., 2009; Skurlova et al., 2011).

In contrast to a number of studies on the roles of inflammation and oxidative stress in pathological anxiety, limited studies have been done to explore the association of anxiety disorders with adaptive immunity

2.2. D. Roles of Immunity in Pathogenesis of Anxiety

Immune protections consist of both innate and adaptive immune responses. Inflammation is considered a part of the innate immune response, while B and/or T cell mediated immune response is considered an adaptive immune response. One study reported that higher anxiety responses are linked with cell mediated immunity. Academic examination stress in students as manifested by anxiety behavior was linked to decreased lymphocyte proliferation in response to mitogens (10 µg/ml concavalin) in students (Wadee et al., 2001).

In conclusion, oxidative stress and inflammation play important roles, though inconsistent, in the pathogenesis of anxiety disorders. More investigation is required before a complete understanding of the roles of oxidative stress, inflammation and systemic immunity in anxiety.

2.3. Currently Available Treatment for Anxieties and the Need for Alternative Therapies

There are a range of pharmacological and non-pharmacological therapies available to treat anxiety. Various pharmacological therapies are 1.) benzodiazepines, 2.) barbiturates, 3.) tricyclic antidepressants, 4.) selective norepinephrine reuptake inhibitors, 5.) selective serotonin reuptake inhibitors, 6.) monoamine oxidase inhibitors, and 7.) serotonin antagonists. There are numerous adverse effects and significant drug-interactions with the current therapies. Benzodiazepines (first line of therapy) have severe adverse effects such as tolerance / physical dependence, drowsiness, dizziness, sedation, psychomotor impairment, memory disruption, vertigo, dysarthria, ataxia, impaired psychomotor function, anterograde amnesia, decrease in blood pressure / cardiac output, weight gain, paradoxical anxiety or aggression, risks of accidents and even mortality (Lader, 1999) (Table 2.2). Furthermore, rapid withdrawal of benzodiazepine can decrease GABA type A receptors activities and can increase the risk for seizure.

Table 2.2. Currently available therapies to treat anxiety disorders (A) and their most common adverse effects (B).

A

Classes of Anxiolytic Drug

- Benzodiazepines
- Barbiturates
- Tricyclic antidepressant
- Selective norepinephrine reuptake inhibitors
- Selective serotonin reuptake inhibitors
- Monoamine oxidase inhibitors
- Serotonin antagonists

B

Common Adverse Effects

- Tolerance
- Physical dependence
- Drowsiness
- Dizziness
- Sedation
- Psychomotor impairment
- Memory disruption
- Vertigo
- Dysarthria
- Ataxia
- Impaired psychomotor function
- Anterograde amnesia
- Decrease in blood
- Weight gain
- Paradoxical anxiety or aggression
- Risks of accidents and even mortality

Selective serotonin reuptake inhibitors (SSRIs) and serotonin-norepinephrine reuptake inhibitors (SNRIs) are ineffective and exhibit a lack of response in many patients (Katzman, 2009). Based on limited knowledge on pathogenesis of anxiety, it is obvious that multiple factors contribute pathogenesis of anxiety disorders. However, most of currently available therapeutics to treat anxiety diseases are treat the symptoms and are monomodal in their action. Therefore, there is a need to explore novel multifunctional therapies that act via a range of mechanisms in neurons with maximal therapeutic efficacy and minimal adverse effects to treat neuropsychological diseases like anxiety disorders. In this regards, certain chemicals in medicinal plants contain a range of bioactive chemicals are potential candidates to treat various neuropsychological disorders

2.4. Alternative Anxiolytic Herb *Scutellaria lateriflora* and Biological Activities of its Flavonoids

Plants of the *Scutellaria* genus are rich in a variety of active chemical compounds, including flavonoids (e.g., wogonin, baicalein etc.) that exhibit a range of biological activities (Choi et al., 2005; Kim et al., 2009). Flavonoids are a group of plant secondary metabolites known to have a range of biological activity. Bergeron et al. (2007), determined flavonoid and amino acid concentrations in an alcohol extract of *Scutellaria lateriflora* plant. They reported that aerial parts of *S. lateriflora* contain principally flavonoid glycosides, with baicalin as the major form, followed by dihydrobaicalin, lateriflorin, ikonnikoside I, scutellarin, oroxylin A-7-*O*-glucuronide, baicalein and wogonin. Total flavonoid content was about 23% of the dry weight of the extract (Bergeron et al., 2005). Out of these chemicals, baicalin and dihydrobaicalin content were found to comparise 14% and 4%, respectively of the ethanol extract. GABA is the major neurotransmitter in this extract, followed by glutamine constituting approximately 0.55% and 0.34% of dry weight, respectively. GABA was thought to be responsible for the anxiolytic action (Bergeron et al., 2005). Despite presence of various bioactive compounds, limited scientific studies has been done of the *Scutellaria lateriflora* (American skullcap) herb.

2.4. A. *Scutellaria lateriflora* Flavonoids and their Anti-inflammatory Effects

Neuro-inflammatory processes are known to contribute to a cascade of events resulting in neurodegenerative disorders, such as anxiety and Alzheimer's disease (Spencer et al., 2012). Recently, there has been more interest in the potential neuroprotective effects of flavonoids. With respect to the nervous system, flavonoids are highly effective in preventing neuro-degeneration in both animals and humans. The anti-inflammatory activities of baicalein contribute to the neuroprotective effects. Rats subjected to controlled cortical impact injury when injected with baicalein (30 mg kg-1) immediately after injury, resulted in

improved functional recovery and reduced the number of degenerating neurons (31%) within 24 hours post-injury. These changes were associated with significantly decreased levels of pro-inflammatory cytokines TNF-α, IL-1β and IL-6 mRNA in brain tissue (Chen et al., 2008).
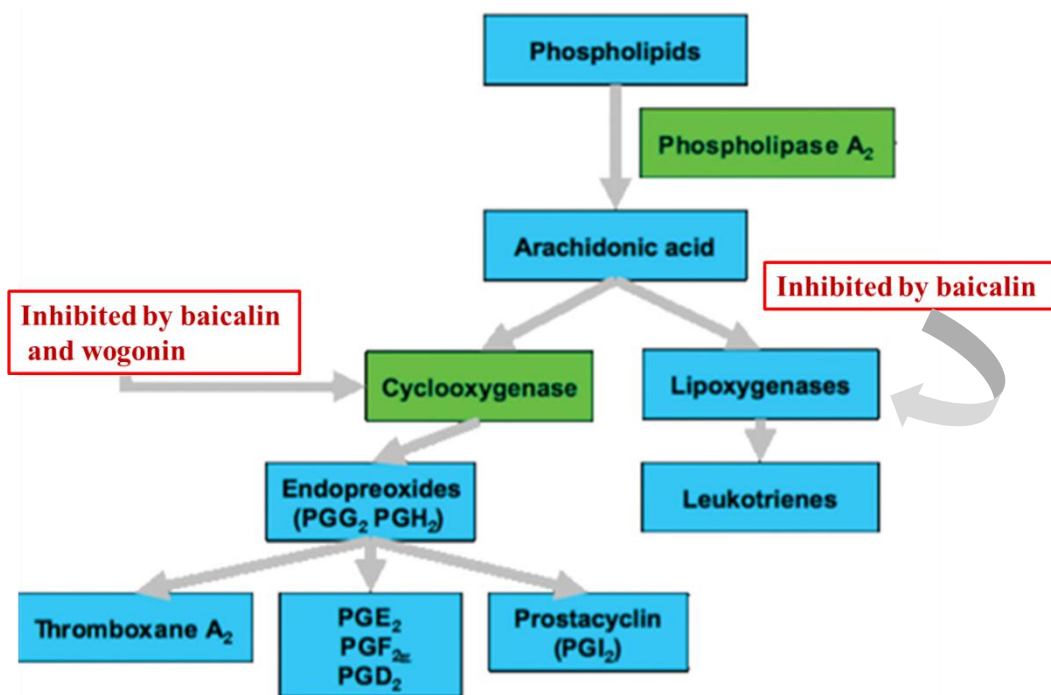
Figure 2.3. Anti-inflammatory activities of *Scutellaria lateriflora* flavonoids.

Liu et al., 2010 investigated the neuroprotective effects of baicalein in Sprague-Dawley rats subjected to permanent middle cerebral artery occlusion (MCAO) to induce cerebral ischemia. Baicalein was administered via i.v. route immediately after inducing cerebral ischemia. Twenty four hours after the MCAO, neuronal deficit were measured by brain water content and infarct sizes. Baicalein was found to reduce brain water content and infarct sizes, and downregulated the overexpression of 12/15-LOX and phospholipase A (cPLA2), measured after MCAO. The results indicated that baicalein protected the brain from damage caused by cerebral ischemia through the downregulation of the proinflammatory enzymes 12/15-LOX and cPLA2 (Liu et al., 2010). (Figure 2.3)

Baicalein also exerts potent neuroprotective effect on LPS-induced injury of dopaminergic neurons (Li et al., 2005). To test the potential neuroprotective effect of baicalein on dopaminergic neurons, primary midbrain neuron-glial cultures were pretreated with baicalein for 30 min prior to stimulation with LPS (10 ng/mL). Morphological studies show that baicalein (5 μM) completely blocked LPS-induced activation of microglia. Baicalein suppressed excessive production of TNFα and free radicals (such as nitrous oxide and superoxide) induced by LPS stimulation in a concentration-dependent pattern (Li et al., 2005). Oxygen and glucose deprivation leads to neuron cell death via apoptosis and suppressed toll like receptor-2 (TLR-2) expression, in neurons (Li et al., 2012a). Baicalin, baicalein, wogonoside, and wogonin exhibited protective effects on oxygen and glucose deprived neuron and increased TLR-2 protein expression (Li et al., 2012a). Nakamura et al., 2003 investigated the effects of baicalin, baicalein, and wogonin on IL-6 and IL-8 protein production, mRNA expression, and NF-kB binding activities induced by IL-1β in human retinal pigment epithelial cell lines (ARPE-19). IL-1β was added to a serum-free medium of ARPE-19 cells to induce IL-6 and IL-8 mRNA expression and protein levels, then co-incubated with a range of concentrations of flavonoids. IL-1β increased IL-6 and IL-8, in the culture media of ARPE-19 cells, in a dose-dependent manner. Dexamethasone, baicalein, and wogonin significantly suppressed IL-6 and IL-8 production, but, baicalin did not suppress IL-1β-induced IL-6 and IL-8 production. Furthermore, increased expression of IL-6 and IL-8 mRNA was significantly suppressed by dexamethasone, baicalein, and wogonin, respectively, but not baicalin (Nakamura et al., 2003). The binding activities of NF-kB, a transcription factor, to IL-6 and IL-8 promoters were measured by an electrophoretic mobility shift assay. It was found that NF-kB binding activities were not suppressed by baicalin and baicalein, but was suppressed by wogonin. The results indicate that wogonin may inhibit IL-6

and IL-8 mRNA expression via the suppression of NF-kB binding activities (Nakamura et al., 2003).

The results of these studies suggest that flavonoids present in *Scutellaria lateriflora* exhibits anti-inflammatory activities via suppressing pro-inflammatory cytokines (Table 2.3).

Table 2.3. Biological activities of *Scutellaria lateriflora* Flavonoids.

Compounds	Anti-inflammatory	Anti-oxidation	Immune response
Bacialin	+	+	+
Bacialein	+	+	+
Wogonin	+	+	+
Chyrsin	+	+	+
Wogonoside	+	+	

Note: + sign indicates the stimulatory effects on the response or activity.

2.4. B. Immunomodulatory Activities of *Scutellaria lateriflora* Flavonoids

The immune system plays important roles to limit the range of adverse affects caused by infections. Studies have shown that plants of the genus *Scutellaria* contain a range of immunomodulatory bioactive compounds (Shang et al., 2010). Interleukin-12 (IL-12) and IFN-γ are the two important cytokines produced by innate immune cells that play important roles to induce cell mediated immune response. IFN-γ and IL-12 both stimulate cell mediated immunity by stimulating Th1 (CD4+ and CD8+ T cells). When plant extracts containing baicalein and/or wogonin stimulate both IL-12 and/or IFN-γ production from leukocytes (Blach-Olszewska et al., 2008; Lim, 2004). Furthermore, wogonin stimulates lymphocytic proliferation (Ohtake et al., 2005) (Figure 2.4).
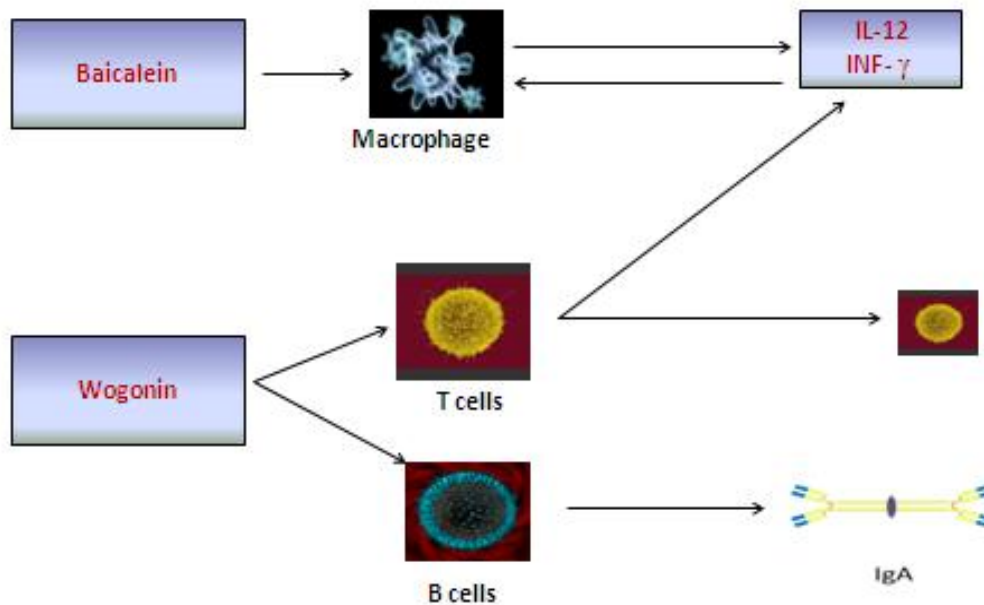
Figure 2.4. Immunomodulatory activities of *Scutellaria lateriflora* flavonoids.

Enhancing gut IgA concentration by American skullcap flavonoid, would conceivably improve protection against infection (Lim, 2004). It has been reported that the flavonoid profile of the *Scutellaria lateriflora* extract is correlated with apoptosis-induction and immunomodulatory activities (Abstracts of Papers, 231st ACS National Meeting, Atlanta, GA, United States, March 26-30, 2006 Joshi et al., 2006). Transforming growth factor beta1 (TGF-β1) is a cytokine secreted from various immune cells including macrophage, T cells and B cells. It can increase or decrease the secretion and activity of other cytokines including IFN-γ, TNF-α and various interleukins. It can also decrease the expression levels of cytokine receptors, such as the IL-2 receptor to down-regulate the activity of immune cells. However, TGF-β1 can also increase the expression of certain cytokines in T cells and promote their proliferation, particularly if the cells are immature immune cells. TGF-β1inhibits proliferation and stimulates apoptosis of B cells. Additionally, it plays a role in class

switching of IgA antibody, transferrin and MHC class II proteins, on immature and mature B cells (Lebman and Edmiston, 1999; Letterio and Roberts, 1998). Wang et al. (2006) incubated different concentrations of bacialin, bacicalein, wogonin and chyrsin with macrophage (RAW 264.7) cells and determined the effects of these flavonoids on TGF-β1 cytokine secretion. It has been found that baicalin, baicalein and chrysin up-regulate TGF-β1 gene expression on macrophage (RAW 264.7) cells in a concentration-dependent manner. However, wogonin, did not up-regulate TGF-β1 expression on gene and protein levels in RAW264.7 macrophage cells (Wang et al., 2006) .

2.4.C. Anti-oxidative Activities of *Scutellaria lateriflora* Flavonoids

Out of 55 herbs tested for antioxidative propertie*s, Scutellaria lateriflora* has one of the highest radical-scavenging activities (Wojcikowski et al., 2007) . The concentration required of baicalein or wogonin glucuronides to scavenge 50% hydroxyl and 1,1-Diphenyl-2-picryl-hydrazyl (DPPH), a stable free radical, was found to be significantly less compared to the known antioxidant ascorbic acid indicating potent antioxidative property of American skullcap (Bochořáková et al., 2003).

Ng et al. (2000) tested the antioxidative activities of baicalin as reflected in the ability to inhibit lipid peroxidation in rat brain and kidney homogenates and rat erythrocyte hemolysis. They found that baicalin exhibited potent antioxidative activity in both lipid peroxidation and hemolysis assays (Ng et al., 2000). Furthermore, Guo et al. (2011) determined that baicalin scavanged DPPH radicals in a dose dependent manner. They further determined the effect of baicalin on xanthine oxidase (XAO) activity by measuring uric acid production in the XAO system. They found that baicalin inhibits XAO activity in a dose dependent manner (Guo et al., 2011). Interestingly, researchers found that baicalin increased anti-oxidative enzymes activities in diseases where oxidative stress plays an important roles in the pathogenesis of diseases (Guo et al., 2011). Viduranga et al. (2011) administered 120

mg/kg of baicalin by oral route for 30 days in type 2 diabetic Goto-Kakizaki rats. It has been found that baicalin-treated type 2 diabetic Goto-Kakizaki rat groups had statistically significant increases ($p < 0.05$) in the activity and expression of the antioxidant enzymes SOD, CAT and GPX when compared with vehicle -treated groups (Waisundara et al., 2011). Further complementing the increase in antioxidant enzyme activity, the oxidative stress markers of plasma lipid peroxide and protein carbonyl contents were reduced in these groups as well (Waisundara et al., 2011). Cao et al. (2011) explored the anti-oxidative activities of baicalin in gerbils subjected to a transient global cerebral ischemic-reperfusion injury. They administered baicalin at doses of 50, 100 and 200 mg/kg via i.p. route to the gerbils immediately after cerebral ischemia and determined the anti-oxidative status of the hippocampus after seven days of cerebral reperfusion (Cao et al., 2011). They found that baicalin at doses of 100 and 200 mg/kg significantly suppressed ischemia-induced neuronal cell damage when determined by hematoxylin and eosin (HE) staining. Baicalin-treated gerbils had reduced levels of malondialdehyde and elevated activities of SOD and GSH as well as GSH-PX in the hippocampus (Cao et al., 2011). Further investigation demonstrated that treatment with baicalin remarkably promoted the expression of the antioxidant BDNF and inhibited the expression of caspase-3 at mRNA and protein levels (Cao et al., 2011). The non-glycosidic form of baicalin, is known as baicalein and also exhibited potent anti-oxidative activities. Reactive oxygen scavenging activities of baicalein was investigated by Park et al. (2003). It has been found that baicalein suppressed reactive oxygen species generation compared to vehicle control in human erythrocytes and its IC_{50} value was determined to be 34.0 μM, which was significantly lower than the IC_{50} value of L-ascorbate and α-tocopherol (Park, 2003) .

2.5. Conclusion

Oxidative stress and inflammation, play important roles in the pathogenesis of neuropsychological and neurodegenerative diseases. *Scutellaria lateriflora*, a native plant of North America, contains a range of bioactive phytochemicals. These, phytochemicals either in pure form or extracted from other plants of genus *Scutellaria,* modulate immune function and, suppress inflammation and oxidative stress. However, despite of having a range of chemicals, limited studies have been done to explore the biological activities of *Scutellaria lateriflora*.

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3. Anti-oxidative and DNA Protecting Effects of Flavonoids-rich *Scutellaria lateriflora*

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The number of figures: 04; The number of tables: 01

Supplementary online materials: No

Running title: Anti-oxidative effects of flavonoids-rich *Scutellaria lateriflora*

Abstract

Scutellaria lateriflora (American skullcap), a native plant of North America, has been used by Americans and Europeans as a nerve tonic for more than 200 years. *In vivo* studies have shown anxiolytic activity of *S. lateriflora* in animals and humans. However, the neuroprotective mechanisms of *S. lateriflora* are not fully understood. Oxidative stress plays a vital role in the neurodegenerative and neuropsychiatric diseases such as anxiety, Alzheimer's disease, depression, and Parkinson's disease. Bioactive compounds present in various medicinal plants neutralize or scavenge toxic free radicals and thus suppress oxidative stress. Therefore, the objective of this study was to investigate the antioxidant effects of *S. lateriflora*. The antioxidant potential of aqueous or ethanolic extracts of *S. lateriflora* was determined in mouse brain tissue using various biochemical assays. Protective effects of *S. lateriflora* against oxidative stress induced DNA fragmentation was determined using plasmid DNA. The ethanolic and aqueous extracts scavenged the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals. The ethanolic extract reduced tert-butyl peroxide-induced reactive oxygen species (ROS) and lipid peroxides in the mouse brain homogenates. Furthermore, the ethanolic extract of *S. lateriflora* protected hydrogen peroxide-UV induced cleavage of supercoiled plasmid DNA. In conclusion, *S. lateriflora* exhibited significant antioxidant effects. The current findings posit *S. lateriflora* as one of the potential experimental herbal drugs that should be screened for its therapeutic potential against various oxidative stress associated mental disorders.

3.1. Introduction

Oxidative stress in the brain is a potential pathogenesis for behavioral deficits and neurological disorders (Ng et al., 2008). Excessive reactive oxygen species (ROS) generation leads to protein and DNA structural alterations, inflammation, tissue damage and subsequent cellular apoptosis or necrosis. An oxidative stress environment in a cell is strictly controlled by either antioxidant molecules (ascorbic acid or α- tocopherol) or antioxidant enzymes such as CAT or SOD. Alterations of antioxidant molecules or enzymes have been associated with the pathogenesis of a range of neurological disorders including Alzheimer's and Parkinson's diseases, anxiety in human patients suffering from obsessive–compulsive disorder and panic disorder (Bouayed et al., 2009; Uttara et al., 2009).

Natural antioxidants are being considered as prospective therapeutic agents and nutritional supplements against neuronal loss. Bioactive compounds (phytochemicals) present in medicinal plants neutralize or scavenge unstable and toxic free radicals (Tumbas et al., 2012) thus, preventing them from attacking vital components (DNA, protein, mitochondria, and lipids) of cells. *Scutellaria* species are rich in a variety of active chemical compounds including flavonoids (wogonin, baicalin, baicalein, chrysin), exhibiting a wide range of biological activities (Shang et al., 2010). Out of approximately 200 species of *Scutellaria*, American skullcap (*S. lateriflora*) and Chinese skullcap (*S. baicalensis*) are officially recognized sources of herbal products of medicinal and nutritional values. *S. lateriflora*, a native plant of North America, has been used by Americans and Europeans for its anti-anxiety, sedative and anticonvulsant properties for several centuries. Aerial parts of *S. lateriflora* have been reported to possess dose dependent anxiolytic effects in a double blind, placebo-controlled study of healthy human subjects (Wolfson and Hoffmann, 2003). GABA constitutes approximately 0.55% of the dry weight of alcoholic extracts of *S. lateriflora* leaves and stem (Bergeron et al., 2005), which is considered to be responsible for its

anxiolytic activity (Sarris, 2007; Zhang et al., 2009). In addition, aerial parts of *S. lateriflora* also contain flavonoid glycosides, including baicalin, dihydrobaicalin, lateriflorin, ikonnikoside I, scutellarin, oroxylin A-7-*O*-glucuronide, baicalein and wogonin (Bergeron et al., 2005). It has been reported that bioactive flavonoids present in American skullcap, either in purified form or extracted from other species of *Scutellaria,* modulate GABA receptor function and thereby exhibit anxiolytic activities in animal models (Hanrahan et al., 2011).

Despite the identification of various flavonoids in *S. lateriflora*, there have been limited scientific studies that delineate the other beneficial properties of *S. lateriflora*. Therefore, the objective of this study was to investigate the antioxidant properties of *S. lateriflora*. To accomplish our goal, ethanolic extracts from the aerial parts of *S. lateriflora* were prepared and radical scavenging capacities were determined in mouse brain tissue.

3.2. Materials and methods

Plant material and chemicals: S. lateriflora seeds were obtained from Horizon Herbs LLC (Lot no. 4232, William, OR) and sown in Marvyn loamy sand at the Horticulture Unit of the E.V. Smith Research Center (Alabama Agriculture Experiment Station, Shorter, Alabama). The seeds were sown in the spring of 2007 and the above ground portions of each plant were cut 7.5 cm from the ground using pruning shears during the following summer. Collected aerial parts of plants were placed in open paper bags and dried in a forced-air dryer (Model AA-5460A, Parameter Generation and Control Inc, Black Mountain, NC) at 40 ^{o}C for 3 days. The dried aerial parts of plants were ground to a fine powder using a Wiley Mill (Model 4, Thomas Scientific, Swedesboro, NJ) mill and stored at room temperature in large cloth bags that allowed movement of air. All chemicals, except where noted, were purchased from Sigma-Aldrich (St Louis, MO).

Preparation of ethanolic and aqueous extracts: Ethanolic or aqueous extracts were prepared according to a previously published method (Bergeron et al., 2005). Ground powder was

either mixed in 70% aqueous ethanol or distilled water for 24 h at room temperature on a platform shaker (Model VXR S10, Tekmar technology, Funkentstort, West Germany). Extracts were then centrifuged for 30 min at 1500 rpm and supernatant was filtered through 0.2 μm filter paper, evaporated to dryness and stored at -40 ^{o}C until further use.

Analysis of total protein, glutathione, polyphenol and flavonoid contents: Protein assay was performed using the Coomassie Plus Protein Assay Reagent Kit (Pierce, Rockford, IL) using bovine serum albumin (BSA) as the standard. Glutathione (0.01-1μg) was used as external standard for calculation of glutathione content (Dhanasekaran et al., 2007). Total phenol content was measured using the Folin-Ciocalteu method (Ismail et al., 2004). The total flavonoid content was determined using the previously described method (Quettier-Deleu et al., 2000).

Effect of S. lateriflora *on DPPH radical scavenging activity:* The 2,2-diphenyl-1-picryhydrazyl (DPPH) assay was performed as described previously (Tharakan et al., 2005) . *S. lateriflora* extracts were added to aliquots of 150 μL of 0.004% methanolic solution of DPPH in 96 well plates in such a manner so that the final concentration of the extract ranged from 0-1000 μg/mL. The mixture was shaken vigorously and left to stand at room temperature for 30 min in the dark. The absorbance was measured at 517 nm against a blank using a spectrophotometer. Ascorbic acid was used as a positive control. The absorbance was measured at 517 nm. Inhibition of DPPH free radical (I%) was calculated according to the formula: I%=(Ablank-Asample)/Ablank×100 where *A*blank is the absorbance of the control reaction (containing all reagents except the test compound), and *A*sample is the absorbance of the test compound. The extract concentration providing 50% inhibition (IC$_{50}$) was calculated from the graph plotting inhibition percentage against extract concentration.

Effect of Scutellaria lateriflora on t-butyl peroxide-induced lipid peroxidation in mouse brain homogenate: The ability of *S. lateriflora* to inhibit lipid peroxidation was measured

according to the previously described method (Dhanasekaran et al., 2009). Briefly, ethanolic or aqueous extract were incubated with 150 µL of 0.14% (v/v, in water) t-butyl peroxide (10 mM) and 30 µL of 10% (w/v, in PBS, 6.25 µg/µL protein) mouse brain homogenate for 30 min at room temperature in 2 mL centrifuge tubes. Alpha-tocopherol was used as a positive control (50-1000 µg/mL). The final concentration of the extract in each reaction was 0-1000µg/mL. Then, 100 µL of 20% (w/v, in PBS) trichloroacetic acid (TCA) and 400 µL of 0.5% (w/v, in PBS) thiobarbituric acid (TBA) were added to each tube, and incubated at 80oC for 15 min. After incubation, the tubes were centrifuged at 10,000 rpm for 5 min and 300 µL of supernatant was added to each well of a 96-well plate and absorbance measured at 532 nm. Inhibition of lipid peroxidation (%) by *S. lateriflora* extract was calculated in a similar way as that used to determine DPPH free radical scavenging activity (I%).

Effect of Scutellaria lateriflora on reactive oxygen species generation in mouse brain homogenate: The free radical scavenging ability of skullcap was measured using dichlorodihydrofluorescein diacetate (DCF-DA), which can be oxidized by reactive oxygen species to yield a fluorescent product. The fluorescence intensity is proportional to the ROS levels in brain tissue homogenate. Rat brain tissue was homogenized in phosphate buffered saline (PBS, 10%, w/v, 6.25 µg/µL protein), incubated with or without the extract (0-1000 µg/mL), t-butyl hydroperoxide (1mM) and DCF-DA at room temperature for 1 h. The intensity of the fluorescent product at 460/528 nm was proportional to the ability of *S. lateriflora* to reduce ROS (Dhanasekaran et al., 2009).

Effect of Scutellaria lateriflora on DNA cleavage: These experiments were performed in 20 µL reaction volume containing 2 µg of pBR322 plasmid DNA (dissolved in 5mM PBS, pH, 7.4), 2.5 mM final concentration of hydrogen peroxide and 50-1000 µg/mL final concentrations of extract in 0.65 mL polyethylene micro-centrifuge tubes. These tubes were directly placed on the surface of an UV-Crosslinker (Model 1800; 80000µW at 300 nm) for 5

min at room temperature for irradiation (Wolfson and Hoffmann, 2003). After irradiation, 10 μL of sample was mixed with 2 μL of loading buffer and separated on agarose gels (1.5% w/v in tris-acetate-EDTA buffer) using 0.02% (w/v) ethidium bromide for visualization. DNA bands were measured using a Bio-Rad gel doc EQ-gel documentation system. The proportion of fragmented bands in the absence and presence of *S. lateriflora* is a measure of *S. lateriflora*'s ability to prevent free radical damage to DNA.

Statistical analysis: One-way analysis of variance (ANOVA) followed by multiple comparison test was used for finding statistically significant differences between each mean value at $P \leq 0.05$.

3.3. Results

Anti-oxidative Effects of Flavonoids-richScutellaria lateriflora: Assessment of biochemical constituents revealed ethanolic extracts of *S. lateriflora* to contain higher protein, glutathione, polyphenol and flavonoid contents compared with the aqueous extracts (Table 1). Direct radical scavenging activities of *S. lateriflora* were determined using the DPPH radicals scavenging assay. Both ethanolic and aqueous extracts quenched stable DPPH radicals. The concentrations required to reduce 50% DPPH radicals (IC_{50} value) were determined to be 83 and 774 μg/mL for the ethanolic and aqueous extracts, respectively. Both ethanolic and aqueous extracts were less potent than the positive control, ascorbic acid ($IC_{50} = 6$ μg/mL, Figure 1). Pro-oxidant t-butyl hydroperoxide (1mM) was used to induce lipid peroxide production in mouse brain tissue homogenates. In our study, only the ethanolic extract reduced lipid peroxidation induced by t-butyl peroxide ($p < 0.05$, Figure 2). The concentrations required to reduce 50% (IC_{50} value)) thiobarbituric acid reactive substances (TBARS) formation compared with the vehicle control was determined to be 350 μg/mL for ethanolic extracts compared with the positive control alpha-tocopherol ($IC_{50} = 325$ μg/mL) (Figure 2). The antioxidant effect of *S. lateriflora* based on the reduction of lipid

peroxidation was comparable with that of the well-established and widely used antioxidant, alpha-tocopherol. Oxidative stress is associated with an increased production of total reactive oxygen species. Both the ethanolic extract of *S. lateriflora* and the positive control, ascorbic acid, significantly scavenged t-butyl peroxide induced reactive oxygen species generation (Figure 3, $p <0.05$). The lowest dose of the ethanolic extract of *S. lateriflora* and ascorbic acid scavenged more than 80% ROS generation in the brain tissue homogenate.

DNA Protecting Effects of Flavonoids-rich Scutellaria lateriflora: Lastly, we determined the effect of the flavonoid rich ethanolic extract of *S. lateriflora* on ROS induced DNA damage. Hydrogen peroxide/UV exposure resulted in cleavage of super-coiled plasmid DNA, which was protected by the highest dose (500μg/mL) of the ethanolic extract of *S. lateriflora* (Figure 4).

3.4. Figures and Figures legend

Table 3.1. Total protein, glutathione, flavonoid and polyphenol contents of the ethanolic and aqueous extracts of *Scutellaria lateriflora*

Extract	Protein (μg/mg)	Glutathione (μg/mg)	Polyphenols (μg/mg)	Flavonoids (μg/mg)
Ethanolic	6.88 ± 0.14a	0.27 ± 0.005	47.7 ± 0.007	0.22 ± 0 .001
Aqueous	0.52 ± 0.12	0.19 ± 0.007	10.6 ± 0.01	0.01 ± 0.003

a Each value represents mean ± SEM (n=5).
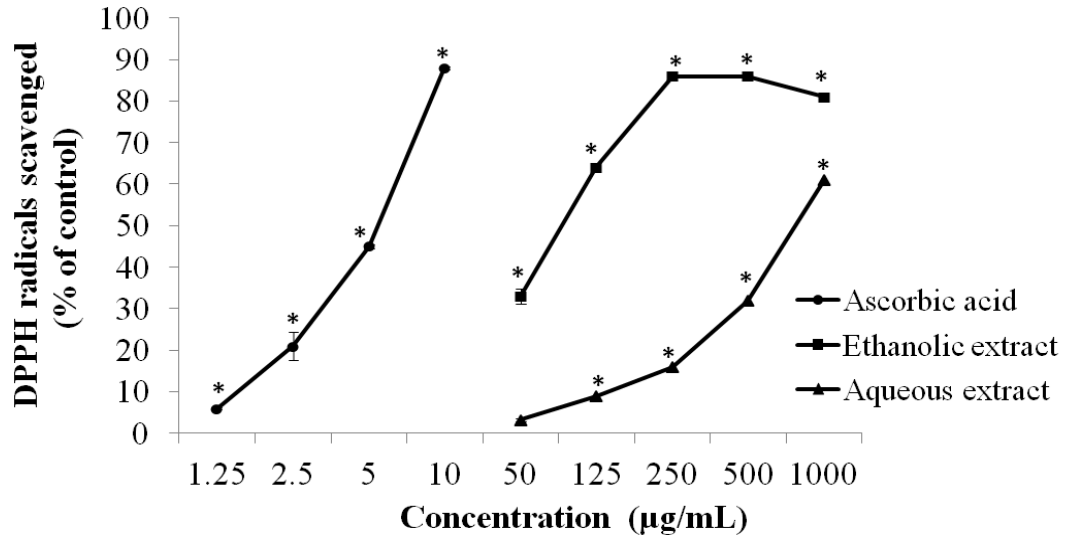
Figure 3.1. 2,2-diphenyl-1-picryhydrazyl (DPPH) radical scavenging effects of ethanolic and aqueous extracts of *Scutellaria lateriflora*. Results are expressed as percentage decrease of absorbance at 517 nm with respect to control (vehicle treated group). Ascorbic acid was used as a positive control. Each value represents the mean ± SEM, (n=6). * $p < 0.05$ indicates significantly different from the control (vehicle treated) group.
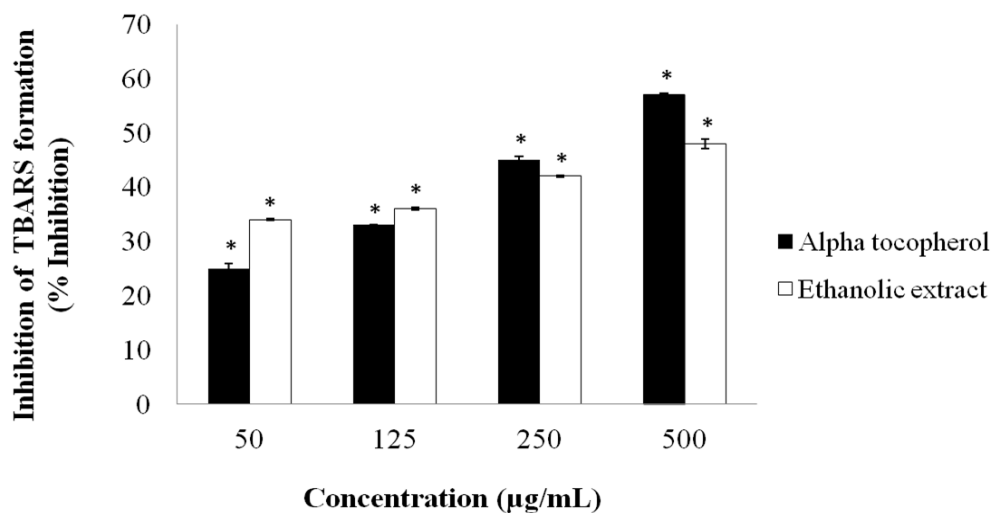
Figure 3.2: Ethanolic extract of *Scutellaria lateriflora* linearly inhibited hydrogen peroxide induced lipid peroxides level generation in mouse brain homogenates relative to vehicle control. α-Tocopherol was used as a positive control for lipid peroxidation generation assays. * $p < 0.05$ indicates significantly different from the control (vehicle treated) group (n=6).
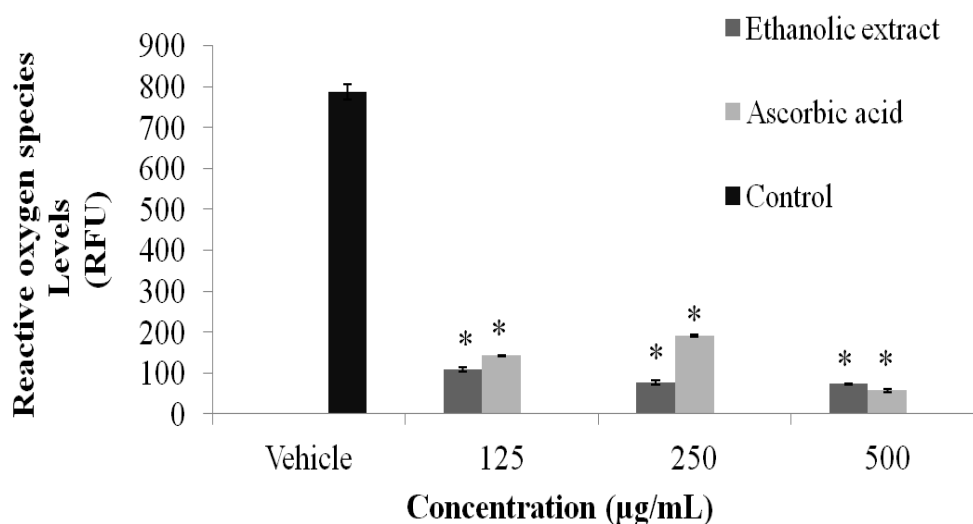
Figure 3.3: Ethanolic extract of *Scutellaria lateriflora* linearly inhibited hydrogen peroxide induced generation of ROS in mouse brain homogenates relative to vehicle control. Ascorbic acid was used as a positive control for ROS generation assays. * p <0.05 indicates significantly different from the control (vehicle treated) group (n=6).
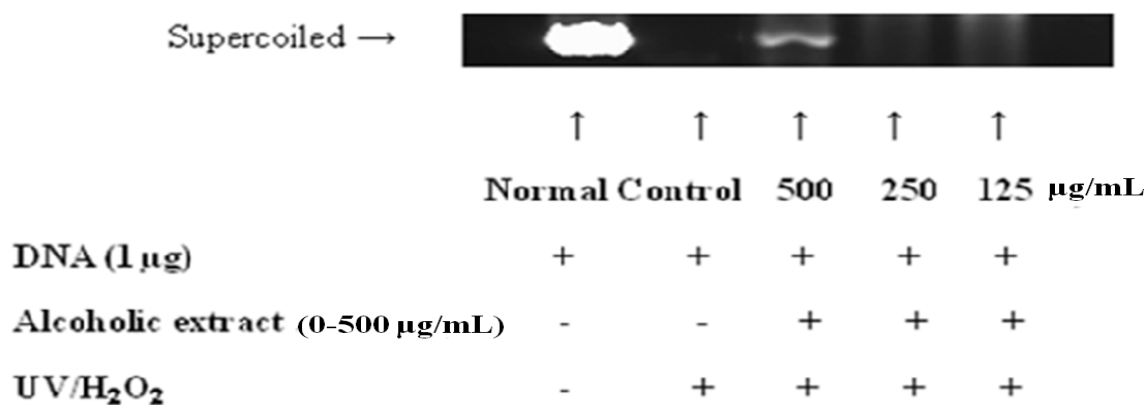
Figure 3.4. Ethanolic extract of *Scutellaria lateriflora* (0-500 µg/mL) protected plasmid DNA (pBR322) against hydrogen peroxide and UV induced cleavage. Experiment was repeated three times. + or − indicates presence or absence of chemicals/compounds, respectively.

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3.5. Discussion

Ayurvedic (Indian), Chinese and South American herbal preparations involving natural phytochemicals have yielded "lead compounds", which have considerably contributed to the current therapies for a wide range of diseases. Moreover, the World Health Organization (WHO) emphasizes the importance of herbal drugs. and their formulation in the therapy of various disease states. Another advantage to the use of herbal medicine as an alternative pharmacotherapy is their lower adverse effects and additional cytoprotective properties as compared with the synthetic drugs intended to treat similar disorders (Tumbas et al., 2012). *S. lateriflora* is a long-standing traditional nervine tonic that has been used for centuries around the world. Interestingly, it is one of the most popular botanicals with many different formulations and products on the USA market (Li et al., 2012b). Hence in this study, we investigated the neuroprotective mechanisms of *S. lateriflora.*

Oxygen is a vital element for neurons, though its imbalanced metabolism and excessive production of oxygen-derived ROS in nerve cells led to toxicity in brain (Bouayed et al., 2009; Halliwell, 2006) . Oxygen containing nucleophilic t-butyl hydroperoxide-induced lipid peroxidation and generation of reactive oxygen species in brain tissue homogenate was inhibited by the ethanolic extract of *S. lateriflora*. The effectiveness of this extract is attributed to the presence of anti-oxidative flavonoids, polyphenols and glutathione. It has been reported that baicalin exhibited potent lipoxygenase inhibitory activity and, therefore, contributes to the lipid peroxidation inhibitory activities of *S. lateriflora*. Polyphenols are the major constituents of *S. lateriflora* that contribute to its antioxidant properties (Chen et al., 2001). Increased production of oxidizing species or decreased capability of the antioxidant defense system during pathogenesis of diseases can result in oxidative stress. Proper folding and functions of major macromolecules, including DNA, are required to maintain homeostasis of a cell. Oxidative stress leads to DNA damage that can lead to cell death

(Tharakan et al., 2005; Uttara et al., 2009). In the present study, *S. lateriflora* protected supercoiled DNA from cleavage induced by hydroxyl radicals generated by UV photolysis of hydrogen peroxide. Our study validated the antioxidant property of *S. lateriflora* based on oxygen radical absorbance capacity (Wojcikowski et al., 2007).

Increased oxygen-derived reactive oxygen species generation due to cellular metabolism in neurons leads to toxicity in various neurological disorders (Bouayed et al., 2009; Halliwell, 2006; Ng et al., 2008). Anxiety is one of the most prevalent neuropsychological disorders worldwide. Approximately, 18 percent of the adult population is affected by anxiety in the USA (Kessler et al., 2005). It has been shown that there is close correlation between oxidative stress and anxiety in human patients suffering from obsessive–compulsive disorder, panic disorder, and demonstrating high trait anxiety (Bouayed et al., 2009). There are several human and animal studies that have implicated the role of oxidative stress in anxiety (Boufleur et al., 2013; Marcolin et al., 2012). Decreased (e.g., ascorbic acid) and / or increased antioxidant CAT activity and SOD levels were observed in the cortex, hippocampus and blood of an animal model of anxiety (Bouayed et al., 2009; Boufleur et al., 2013; Hovatta et al., 2010; Marcolin et al., 2012). Furthermore, vitamin E (tocopheral, lipid soluble antioxidant) deficiency can increase neuropsycological disorders in humans and rodents (Bouayed, 2010; Bouayed et al., 2009; Terada et al., 2010). In general, patients with anxiety disorders have increased generation of ROS, higher lipid peroxidation and DNA oxidation in the brain compared with healthy controls (Hovatta et al., 2010; Kuloglu et al., 2002; Kuloglu et al., 2003; Rammal et al., 2008). Effective use of dietary antioxidants has been reported to exhibit a cognitive enhancing effect, psychostimulant activity, and antidepressant properties (Bouayed, 2010; Bouayed et al., 2009). Therefore, antioxidant rich dietary herbal medicine presents a potential effective and novel approach to the treatment of a range of neurological disorders (Dhanasekaran et al., 2007). In conclusion, *S. lateriflora*

exhibits potent antioxidant effects that reasonably may explain, at least in part, its anecdotal

anxiolytic effects exhibited in experimental animals and in humans.

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4. Neuroprotective mechanisms of *Scutellaria lateriflora*

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The number of figures: 05; The number of tables: 00

Supplementary online materials: No

Running title: Neuroprotective mechanisms of *Scutellaria lateriflora*

In preparation for publication: Oxidative Medicine and Cellular Longevity

Abstract

Cellular damage, due to oxidative stress, severely affect different regions of brain in various neurological disorders including Parkinson disease (PD), Alzheimer disease (AD), Huntington disease (HD), epilepsy, ischemic stroke, and amyotrophic lateral sclerosis (ALS). *Scutellaria lateriflora* (American skullcap), a native plant of North America, has been used by Americans and Europeans to treat neuropsychological disorders. The objective of the current study is to further investigate the neuroprotective properties and mechanisms of *S. lateriflora*. The antioxidant and anti-apoptotic potential of *S. lateriflora* were evaluated in hydrogen peroxide-induced oxidative stress in differentiated hippocampal H19-7 cells. Furthermore, the effect of *S. lateriflora* on brain-derived neurotrophic factor (BDNF) and glutamatergic receptor expression was also measured. *S. lateriflora* extract suppressed caspase-3 expression and scavenged the reactive oxygen species (ROS) of hydrogen peroxide induced oxidative stress. Decreased ROS and caspase-3 activity by *S. lateriflora* extract strongly correlated with the increased cell viability. Additionally, *S. lateriflora* increased BDNF expression in H19-7 cells but did not affect phosphorylated cAMP response element-binding protein (pCREB) or N-methyl-D-aspartate (NR2A and NR2B) receptors expression. The findings indicate that *S. lateriflora* exhibit neuroprotection by increasing nerve growth factor expression and by its antioxidant and anti-apoptotic effects on neuronal cells.

4.1. Introduction

Neurodegenerative diseases are often related to the high metabolic rate and reduced regenerative capacity of neurons and result in part from the brain's inherent susceptibilites to the adverse effects of oxidative stress caused by excessive ROS generation. Oxidative stress is a consequence of an imbalance between the pro-oxidant and antioxidant systems and is reported as a major contributor to neuronal apoptosis which leads to a range of neurodegenerative disorders. Increased levels of oxidative stress related damage have been reported in affected brain regions in various neurological conditions including Parkinson disease (PD), Alzheimer disease (AD), Huntington disease (HD), epilepsy, ischemic stroke, and amyotrophic lateral sclerosis (ALS) (Uttara et al., 2009). Among neuropsychological diseases, anxiety disorders are one of the prevalent problems around the world, affecting 18 percent of the United States population. There has been a close correlation between oxidative stress and anxiety disorders in patients suffering from obsessive–compulsive disorder and panic disorder (Bouayed et al., 2009). In addition to oxidative stress, inflammation, or other factors also contribute to the complex pathophysiology and etiology of neurodegenerative and neuropsychological disorders. The use of multifunctional pharmaceuticals which can act via a range of mechanisms in neurons are required to treat these disorders. However, most of currently available therapeutics used to treat neurodegenerative or neuropsychological function to treat symptoms and are monomodal in their action. Therefore, there is a need to explore novel multifunctional therapeutics that act via a range of mechanisms in neurons to treat neurodegenerative diseases.

Phytochemicals extracted from plants contain a range of bioactive chemicals that are potentialy therapeutic candidates to treat a range of neuropsychological disorders. Plants of *Scutellaria* genus are rich in a variety of active chemical compounds including flavonoids (e.g., wogonin, baicalein etc.) that exhibit a variety of biological activities (Choi et al., 2005;

Kim et al., 2009; Lohani et al., 2013b; Shang et al., 2010). Flavonoid glycosides, including baicalin, dihydrobaicalin, lateriflorin, ikonnikoside I, scutellarin, oroxylin A-7-*O*-glucuronide, baicalein and wogonin are the major known bioactive chemicals present in aerial parts of *S. lateriflora* (Bergeron et al., 2005). Phytochemicals usually act to neutralize or scavenge unstable reactive free radicals (Fiorentino et al., 2009; Min et al., 2011; Sinha et al., 2012; Tumbas et al., 2012) thus preventing them from attacking vital components of cells. *S. lateriflora*, a native plant of North America, has been used by Americans and Europeans for its anti-anxiety, sedative and anticonvulsant properties for more than 200 years.

Results of research studies suggest potential neuroprotective activities of *S. lateriflora. S. lateriflora* extracts and its purified flavonoids inhibit prion protein (PrP) replication *in vitro* and delay the onset of prion disease in mice. Therefore, *S. lateriflora* is considered a promising candidate for the development of a new therapeutic drug against Transmissible Spongiform Encephalopathies (TSE). Other neurodegenerative diseases like Alzheimer's (AD) and Parkinson's disease (PD) may also benefit from treatment with antioxidant phytochemicals (Eiden et al., 2012).

S. lateriflora has anticonvulsant activity in rodent models of acute seizures (Zhang et al., 2009). Furthermore, aerial parts of *S. lateriflora* exhibit dose-dependent anxiolytic effects in a double blind, placebo-controlled study of healthy human subjects (Brock et al., 2013; Wolfson and Hoffmann, 2002). Bioactive flavonoids purified or extracted from other species of *Scutellaria* and also present in *S. lateriflora*, either, modulate gamma-aminobutyric acid (GABA) receptor function and exhibits anxiolytic activities in animal models (Hanrahan et al., 2011). Aerial parts of *S. lateriflora* also contain GABA and glutamine, which further contribute to the anxiolytic activity of *S. lateriflora* (Bergeron et al., 2005).

Despite the identification of various bioactive chemicals in *S. lateriflora*, there have been limited scientific studies evaluating the mechanisms of the neuroprotective effects of *S.*

lateriflora. The objective of this study was to investigate the protective effects of *S. lateriflora* against oxidative stress-induced deleterious effects on neurons. To accomplish this goal, ethanolic extracts from the aerial parts of *S. lateriflora* were prepared and used to determine the molecular mechanisms of protective activity against the detrimental effects of oxidative-stress in hippocampal (H19-7) and pheochromocytoma (PC12) cells.

4.2. Materials and Methods

Plant material and chemicals: S. lateriflora seeds were obtained from Horizon Herbs LLC (Lot no. 4232, William, OR) and sown in Marvyn loamy sand at the Horticulture Unit of the E.V. Smith Research Center (Alabama Agriculture Experiment Station, Shorter, Alabama). The seeds were sown in the spring of 2007 and the above ground portions of each plant were cut 7.5 cm from the ground using pruning shears during the following summer. Collected aerial parts of plants were placed in open paper bags and dried in a forced-air dryer (Model AA-5460A, Parameter Generation and Control Inc, Black Mountain, NC) at 40 ^{o}C for 3 days. The dried aerial parts of plants were ground to a fine powder using a Wiley Mill (Model 4, Thomas Scientific, Swedesboro, NJ) mill and stored at room temperature in large cloth bags that allowed movement of air. All chemicals, except where noted, were purchased from Sigma-Aldrich (St Louis, MO).

Preparation of alcoholic extracts of S. lateriflora. The highest concentration of plant flavonoids was extracted in 70% ethanol as compared to other solvents (Lohani et al., 2013b). Alcoholic extracts of *S. lateriflora* (SLE) was used to determine the mechanisms of neuroprotective activities. Ground, dried powder of *S. lateriflora* leaves and stems was either mixed in 70% aqueous ethanol or distilled water for 24 hours at room temperature on a platform shaker (Model VXR S10, Tekmar technology, Funkentstort, West Germany). The samples were centrifuged for 30 min at 1500 rpm and the supernatant (alcoholic extract)

collected, filtered through 0.2 μm filter paper, evaporated and dried. Dried extract was stored at -4 ^{o}C until further use.

Neuronal Cell Cultures. Embryonic hippocampal neuronal cells (H19-7) was obtained from American Type Culture Collection ((CRL-2526), Manassas, VA). Embryonic hippocampal neuronal cells (H19-7) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 50 units/ml penicillin, 50 units/ml streptomycin, 200 μg/ml gentamicin, and 1 μg/ml puromycin in flasks coated with 15 μg/ml poly-L-lysine. Cells were equilibrated in a humidified atmosphere of 5% CO_2 and 95% O_2 at an air temperature of 34˚C. Cells were propagated up to 80% confluency in 75 cm^2 poly-L-lysine (15 μg/ml in Phosphate Buffered Saline, PBS) coated tissue culture flasks. After reaching 80% confluence, H19-7cells were incubated at 39 ^{o}C for 24 hours in DMEM supplemented with 1% (v/v) FBS and penicillin, streptomycin, puromycin, gentamicin (at concentration listed above), 1% (v/v) Neuro-2 supplement and 50 ng/ml basic fibroblast growth factor were added to media to facilitate for differentiation. Pheochromocytoma cell (PC12) were propagated in RPMI 1640 media containing 10% FBS, 100 units/ml penicillin and 100 units/ml streptomycin in 75 cm^2 flasks coated with 15 μg/ml (in PBS) poly-L-lysine until cells reached 80% confluency. These cells were equilibrated in a humidified atmosphere of 5% CO_2 and 95% O_2 at an air temperature of 37 ˚C. Pheochromocytoma cells were differentiated by replacing culture media with differentiating media containing 1% FBS, 100 units/ml penicillin, 100 units/ml streptomycin and 50 ng/ml of nerve growth factor for 48 hours at 37˚C.

Determination of toxic concentrations (LD$_{50}$) of the SLE in differentiated neuronal cell lines. Undifferentiated H19-7 or PC 12 cells were propagated to 80-90 % confluency in 10 cm^2 culture plates. After harvesting with 0.25% trypsin (w/v, in PBS), these undifferentiated cells were re-plated onto poly-L-lysine coated 96 well plates at a density of 2 ×10^5 cells/ml. Once

cells reached 80% confluency in the wells, they were allowed to differentiate for 24 and 48 hours for H19-7 cells and PC12 cells, respectively. The differentiated cells were incubated with a range of concentrations (0-500 µg/ml, in PBS) of SLE for 24 hours to assess toxic concentrations of SLE. Cells survival were determined by tetrazolium dye (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described earlier (Dhanasekaran et al., 2006). Briefly, MTT was added at a final concentration of 1 mg/ml to each well. The medium was discarded after 4 hrs of incubation at 37°C and the reduced, insoluble dark blue formazan crystals were dissolved in 100 µl of dimethyl sulfoxide (DMSO). Absorbance was subsequently measured at 570 nm with a reference wavelength of 630 nm using a microtiter plate reader (Synergy HT, Bio-Tek Instruments Inc., Winooski, VT).

Determination of neuro-protective effects of SLE against oxidative-stress induced cell death. Non-toxic concentrations of SLE were used to examine its protective effect against hydrogen peroxide-induced cell death. Cells were plated into poly-L-lysine coated 96 well plates at a density of 2×10^5 cells/ml in culture media. After growing to 80 % confluency, cells were allowed to differentiate by incubating with differentiating media for 24 hours. After 24 hours of exposure to differentiation media, cells were pre-incubated with a range of concentrations of SLE extract for 2 hours, after which hydrogen peroxide (200 µM) or PBS was co-added with SLE for 12 hours. At the end of the incubation period, the MTT assay was performed to determine the percentage of live cells in each well.

Western blot. H19-7 cells were treated with SLE as described above. The cells were harvested by washing with PBS and lysed in a buffer containing 50 mM 2-Amino-2-hydroxymethyl-1,3-propanediol chloride (Tris-Cl, pH 7.4), 150 mM sodium chloride, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, and a combination of protease inhibitors. Lysate were centrifuged at -4 °C for 30 minutes and supernatant were

used for Western blot. Protein concentration in each sample was measured using the Thermo Scientific Pierce 660 nm Protein Assay reagent. Supernatant samples were aliquoted into 50 µg fractions and stored at -70 ºC. On the day of the Western blot, samples were boiled with sample loading buffer for 5 minutes and loaded onto a gradient 4-12% NuPAGE Bis-Tris Gel (Life Technologies, Grand Island, NY) and were electrophoretically transferred onto a polyvinylidene difluoride (PVDF) membrane by using the wet transfer method. Non-specific binding sites on the membranes were blocked with 5% fat-free milk in tris-buffered saline plus 0.1% Tween-20 at pH 7.4 (TBST). The membranes were probed with anti-caspase 3, anti-caspase 9, anti-cytochrome C, anti-NR2A, anti-NR2B, anti-CREB, anti-pCREB and anti-BDNF antibodies along with anti β-Actin (Cell Signaling Technology, Beverly, MA). β-actin was used as loading control. Caspase 3 and caspase 9 are markers of apoptosis and NR2A and NR2B are subunits of glutamate (NMDA) receptors. BDNF, whose expression regulated by CREB transcription factor, is an antioxidant protein molecule. NMDA receptors play important roles in learning and memory. Membranes were washed with TBST (3 times, each for 10 min) and incubated with anti-rabbit or anti-mouse species dependent horseradish peroxidase (HRP)-conjugated secondary antibodies (Cell Signaling Technology, Beverly, MA) for 60 min at room temperature. After incubation with each secondary antibody, membranes were again washed three times for 10 min with TBST. After washing membranes were incubated with chemiluminescent substrate for 5 minutes. Membranes were placed in plastic wrap and inserted in the x-ray film cassette with the protein side oriented up adjacent to X-ray film. The chemiluminescence signals were measured as the band density and quantitated with Quantity One software (Bio-Rad Laboratories, Hercules, CA). The band densities for each sample was normalized to their β-actin signal and reported as a percentage of PBS treated control.

Caspase-3 inhibitory activity of SLE. H19-7 cells were treated as described in previously described section and caspase-3 activity was measured as described previously (Bijur et al., 2000) in 96-well clear bottom plates. Cells were lysed with 100 µl of lysis buffer without protease inhibitors as described in Western blot section. The lysates were collected in micro-centrifuge tubes, sonicated, and centrifuged at 14,000 xg for 10 min at 4 $^{\circ}$C. Protein concentrations in the supernatants were determined using the BCA Protein Assay Reagent Kit (Pierce Chemical Co.Rockford, IL). Briefly, the peptide substrate for caspase-3 enzyme, acetyl Asp-Glu-Val-Asp 7-Amido-4-Methyl Coumarin (AC-DEVD-AMC) was added to each well for a final concentration of 25 ng/µl. Supernatants of cell lysates (20 µg protein) were added to start the reaction. Blanks (no caspase 3, and no AC-DEVD-AMC, only PBS), positive inhibition control (N-Acetyl Asp-Glu-Val-Asp-Aldehyde, only but no caspase 3) and positive control (only caspase 3) were added in each plate. Standard curves were prepared by addition of serial dilutions of caspase 3 (0-0.25 U/well) to wells to achieve a final concentration of 25 ng/µl of AC-DEVD-AMC. Ten microliters of caspase 3 inhibitor (N-Acetyl-Asp-Glu-Val-Asp-Aldehyde) were added to caspase-3 substrate to test assay specificity. All measurements were carried out in triplicate wells. Caspase 3 hydrolyses Ac-DEVD-AMC, which results in the release of the fluorescent 7-amino-4-methylcoumarin (AMC) product. Fluorescence was measured on a fluorescence plate reader at 360 nm excitation and 460 nm emissions. Caspase activity was calculated as [(mean AMC fluorescence from triplicate wells) – (background fluorescence)]/µg of protein.

Quantification of reactive oxygen generation. Hippocampal neuronal cells (H19-7) were allowed to differentiate in 96 well plates as previously described section 4.2. After washing with PBS buffer, these cells were incubated with 10 µM of 2'-7'-DichloroDihyDroFluorescein Diacetate (DCFH-DA) (Sigma Aldrich, St. Louis, MO) in the differentiation medium for 60 minutes. After 60 minutes of incubation, DCFH-DA containing media was removed from the

wells and the cells were washed with PBS. Cells were then pre-incubated for 2 hours with a range of concentrations of SLE in differentiation media for 2 hours. After pre-incubation, hydrogen peroxide (100 μM) was co-incubated with the range of concentration of SLE for 12 hours. The fluorescence of cells from each well was measured by microtiter plate reader (Synergy HT, Bio-Tek instrument, Winooski, VT, USA) at an excitation wavelength of 485 and emission wavelength of 530 nm at 37 $^{\circ}$C.

Antioxidative enzyme activity assays. Catalase activity was measured by the method of Beers and Sizer (1952). In this assay, hydrogen peroxide was used as substrate for catalase and disappearance of hydrogen peroxide was measured spectrophotometrically at 240 nm. Briefly, 40 μl of cell lysate and 260 μl of 30 mM hydrogen peroxide solution in phosphate buffer saline (0.05M, pH 7) were added in a cuvette (Beers and Sizer, 1952). A standard curve was prepared with commercially available catalase (Cayman Chemicals, Ann Arbor, MI). The solution of hydrogen peroxide was added last since hydrogen peroxide will be consumed very fast. Absorbance at 240 nm was measured before and after adding hydrogen peroxide. Catalase activity was measured for 2 minutes by calculating the difference in absorbance per minute. Results are expressed relative to PBS treated controls after normalizing for protein content. The total superoxide dismutase (SOD) activity was measured by using the High Throughput Superoxide Dismutase Kit (Trevigen, Gaithersburg, MD) according to the manufacturer's protocol.

Statistical analysis. Results of the experiments are expressed as mean ± standard error. Statistical analysis was performed by using a one way ANOVA with Dunnet post test. Significance was obtained of $p < 0.05$. All statistical analyses were performed using Prism-V software (GraphPad Software, Inc, San Diego, CA).

4.3. Results

Toxic concentrations of SLE or hydrogen peroxide: A range of concentration of Alcoholic extract of *S. lateriflora* were incubated with differentiated H19-7 and PC12 for 24 hours and 48 hours,respectively. Alcoholic extract of *S. lateriflora* was found to be non-toxic to differentiated H19-7 and PC12 cells up to the concentrations of 100 µg/ml and 125 µg/ml, respectively. The concentrations determined for SLE to kill 50% differentiated H19-7 and PC12 cells were 175 µg/ml and 225 µg/ml, respectively (Figure 4.1 A). Hydrogen peroxide decreased cell viability in a concentration dependent manner when incubated with differentiated H19-7 and PC12 cells for 12 hours and 24 hours, respectively. The concentration required to kill 50% H19-7 and PC12 cells was 150 µM and 200 µM, respectively (Figure 4.1 B).

Protective effects of SLE on H19-7 and PC12 cells against hydrogen peroxide induced oxidative stress: Hydrogen peroxide (200 µM) induced 50% of PC12 cells death (Figure 4.2 A) and 65% of H19-7 cells death (Figure 4.2 B) compared to vehicle treated control group. Similar to the known antioxidant, N-acetyl cystine, 5-20 µg/ml concentration of SLE protected both H19-7 and PC12 cells against hydrogen peroxide induced cell death. (Figures 4.2 A and 4.2 B). Alcoholic extract of *S. lateriflora* enhanced viability of hydrogen peroxide treated H19-7 and PC12 cells up to 80% compared to only hydrogen peroxide treated H19-7 (35% viablility) and PC 12 cells (50% viability). However, lowest (2.5 µg/ml) concentration of SLE did not provide protection against hydrogen peroxide-induced cell death (Figure 4.2 A and 4.2 B). Treatment of H19-7 cells with hydrogen peroxide resulted morphological changes in, such as cell shrinkage, membrane blebbings, rounding of cells and detachment from the bottom of flask. These morphological changes were not observed in majority of SLE treated H19-7 cells exposed to hydrogen peroxide (Figure 4.2 C).

Effects of SLE on reactive oxygen species generation, superoxide dismutase and catalase activities in hydrogen peroxide treated H19-7 cells: Hydrogen peroxide enhanced reactive oxygen species generation in H19-7 cells (Figure 4.3 A). SLE (5-10 µg/ml) suppressed hydrogen peroxide increased reactive oxygen species generation in H19-7 cells compared to positive control (only hydrogen peroxide treated cells) (Figure 4.3 A). Hydrogen peroxide increased both SOD2 and CAT activities in H19-7 cells compared to only vehicle treated H19-7 cells. However, 5-10 µg/ml of SLE further increased SOD2 and CAT activities in hydrogen peroxide exposed cells compared to positive control (only hydrogen peroxide treated cells) (Figure 4.3 B and 4.3 C)

Effects of SLE on pro-apoptotic molecules expression in H19-7 cells: Undifferentiated H19-7 cells after reaching up to 80% confluency were incubated at 39 ^{o}C (undifferentiated cells were were grown at 37 ^{o}C) and 1% FBS (undifferentiated cells were grown using 10% FBS) to stimulate their differentiation. It has been reported that differentiation of cells led to their apoptosis. Therefore we determined anti-apoptotic effects of SLE on differentiated H19-7 cells. Differentiation of H19-7 cells resulted in increased cytochrome C, caspase 9 and caspase 3 protein expressions compared to undifferentiated H19-7 cells (Figure 4.4 A). Flavonoid rich SLE suppressed cytochrome C, caspase 9 and caspase 3 protein expression in differentiated H19-7 cells compared to vehicle treated differentiated H19-7 cells (Figure 4.4 A). SLE suppressed caspase 3 and cytochrome C protein expression in hydrogen peroxide treated differentiated H19-7 cells compared to control (only vehicle treated differentiated H19-7 cells) (Figure 4.4 B). Hydrogen peroxide increased caspase 3 enzyme activity in differentiated H19-7 cells as compared to control (only vehicle treated differentiated H19-7 cells) (Figure 4.4 C). However, SLE suppressed caspase 3 enzyme activity induced by hydrogen peroxide in H19-7 cells compared to positive control (only hydrogen peroxide treated differentiated H19-7 cells) (Figure 4.4 C).

Effects of SLE on neurotrophic growth factor and memory markers expression in hydrogen peroxide treated H19-7 cells. Differentiation of H19-7 cells resulted in increased N-methyl-D-aspartate receptor subunit (NR2A), brain derived growth factor (BDNF) and phosphorylated cAMP response element-binding (pCREB) protein expression compared to undifferentiated H19-7 cells (Figure 4.5 A). Excitatory NMDA receptors and growth factor BDNF play important roles in memory functions. However, protein expression of NR2B subunit of N-methyl-D-aspartate receptor in differentiated H19-7 cells was not modulated compared to undifferentiated cells (Figure 4.5 A). SLE did not affect N-methyl-D-aspartate receptor (NR2A and NR2B proteins), BDNF and pCREB expression in differentiated H19-7 cells compared to control (Figure 4.5 A). Hydrogen peroxide suppressed protein expression of NR2A and NR2B proteins, BDNF and pCREB protein expression in differentiated H19-7 cells compared to control (vehicle treated differentiated H19-7 cells) (Figure 4.5 B). SLE significantly increased BDNF protein expression compared to positive control (only hydrogen peroxide treated cells) (4.5 B). However, SLE did not affect expression of other proteins compared to positive control (4.5 B).

A

B
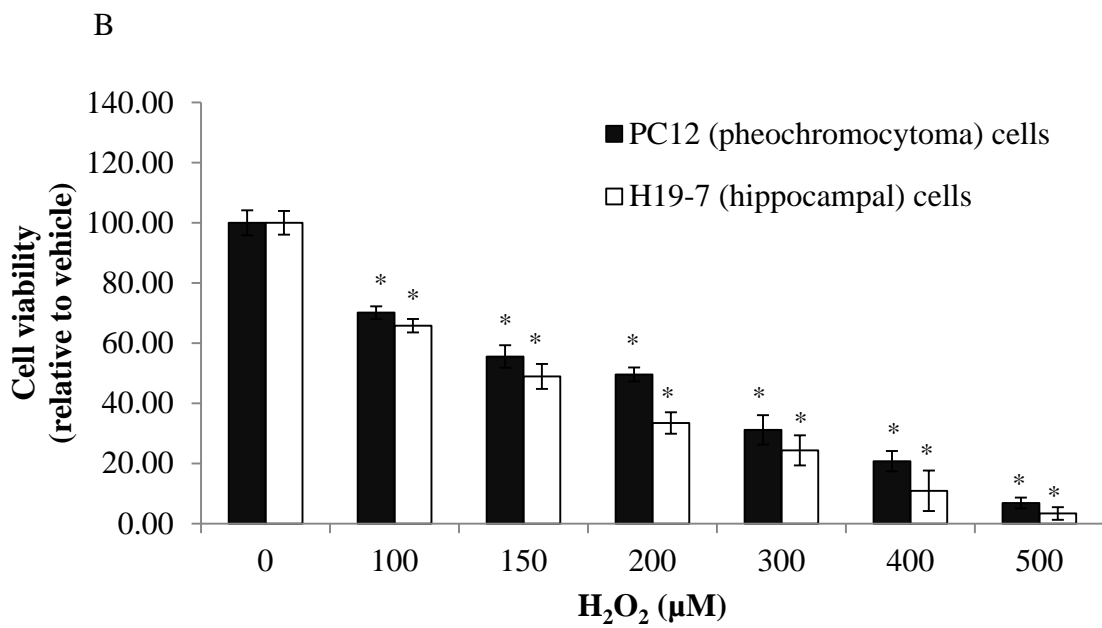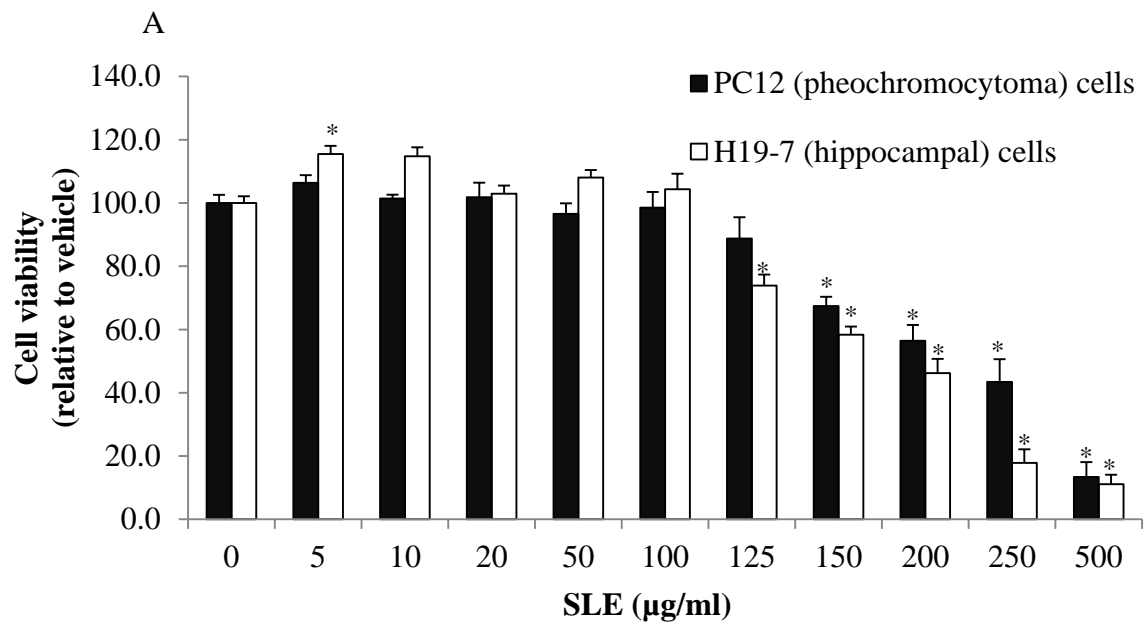
Figure 4.1. Effects of ethanolic extract of *Scutellaria lateriflora* (SLE) and hydrogen peroxide on differentiated hippocampal (H19-7) and pheochromocytoma cell (PC-12) lines. Undifferentiated H19-7 and PC-12 cells were grown in 96 well plates to 70-80% confluency, and allowed to differentiate by incubating in differentiating media for 24 hours (H19-7 cells) or 48 hours (PC-12 cells). Differentiated cells were only incubated with a range of concentrations of SLE (0-500 μg/ml) for the next 14 hours (H19-7 cells) or 24 hours (PC 12 cells) (A, upper panel). Similarly, cells were only incubated with a range of concentrations of hydrogen peroxide (0-500 μM) for 12 hours (H19-7 cells) or 24 hours (PC 12 cells) (B, lower panel). Each value represents the mean ± SEM, (n=6) obtained from 3 independent experiments performed in duplicate. [*] indicates significant difference from control (vehicle treated cells) at $P < 0.05$.
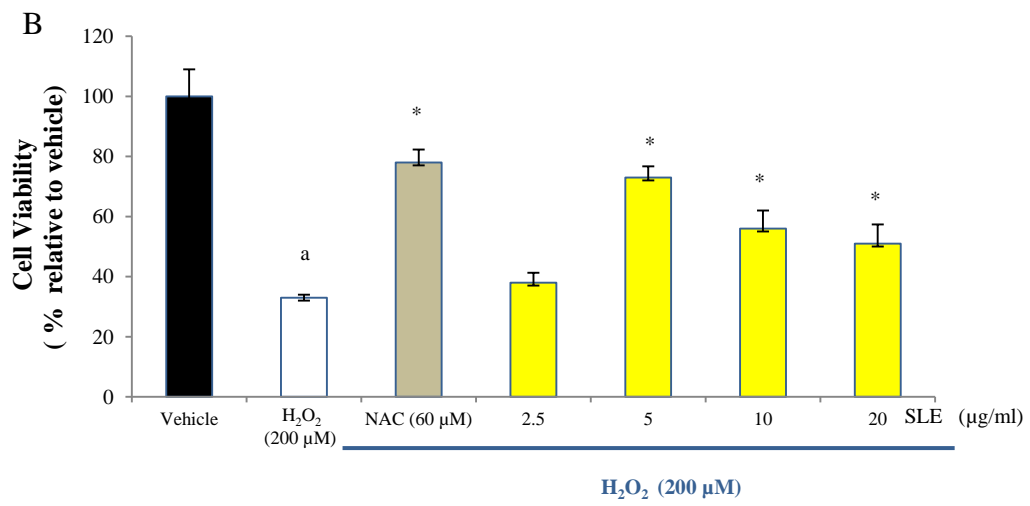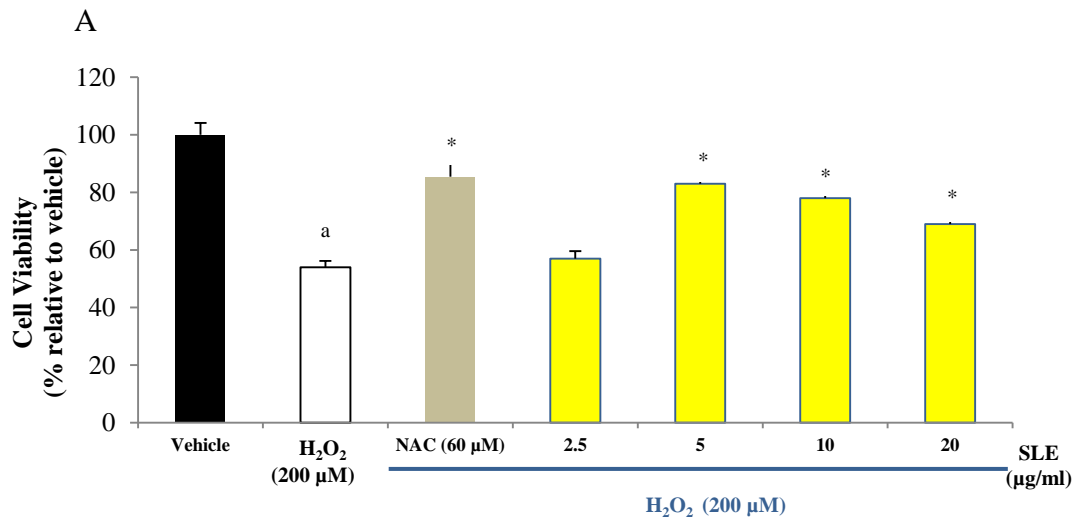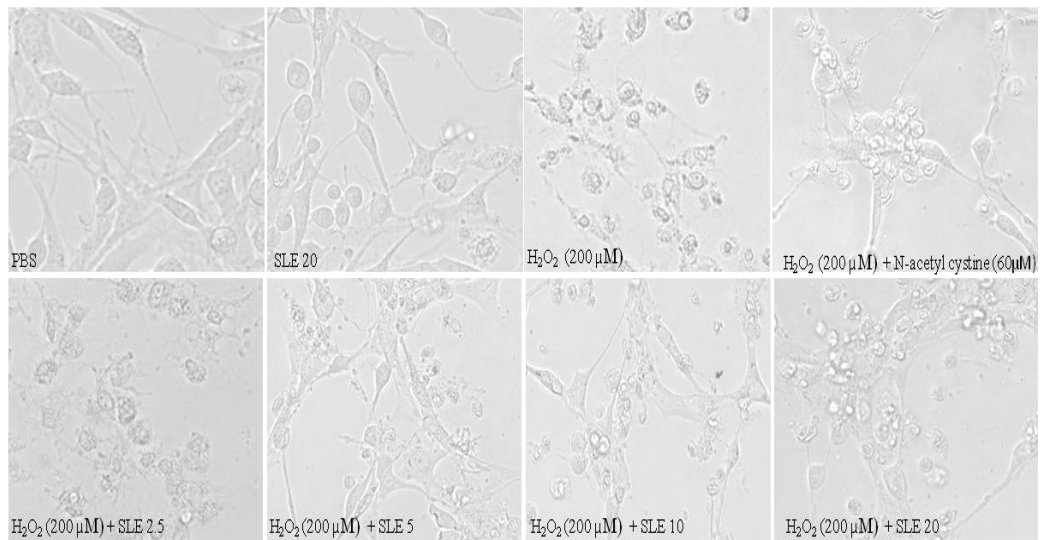
Figure 4.2. Protective effects of SLE against hydrogen peroxide induced oxidative stress in differentiated PC-12 and H19-7 (A and B). Differentiated cells were pre-incubated with various concentration of SLE for 2 hours and the co-incubated with hydrogen peroxide (200 µM) for 12 hours (H19-7 cells) or 24 hours (PC-12 cells). Photographs of H19-7 cells viewed under light microscope after treatment 40X magnification (C). Results are expressed relative to vehicle (phosphate buffered saline) control. Each value represents the mean ± SEM, (n=3) obtained from 3 independent experiments performed in duplicate. N-acetyl cystine (NAC) is an anti-oxidant and act as a positive control.

[a] indicates significant difference from vehicle control (phosphate buffered saline) ($P < 0.05$). * indicates significant difference from positive control (hydrogen peroxide treated cells) ($P < 0.05$). Abbreviation: SLE 5 µg/ml indicates 5 µg dried down ethanol extract of *Scutellaria lateriflora*/ml of cell media.
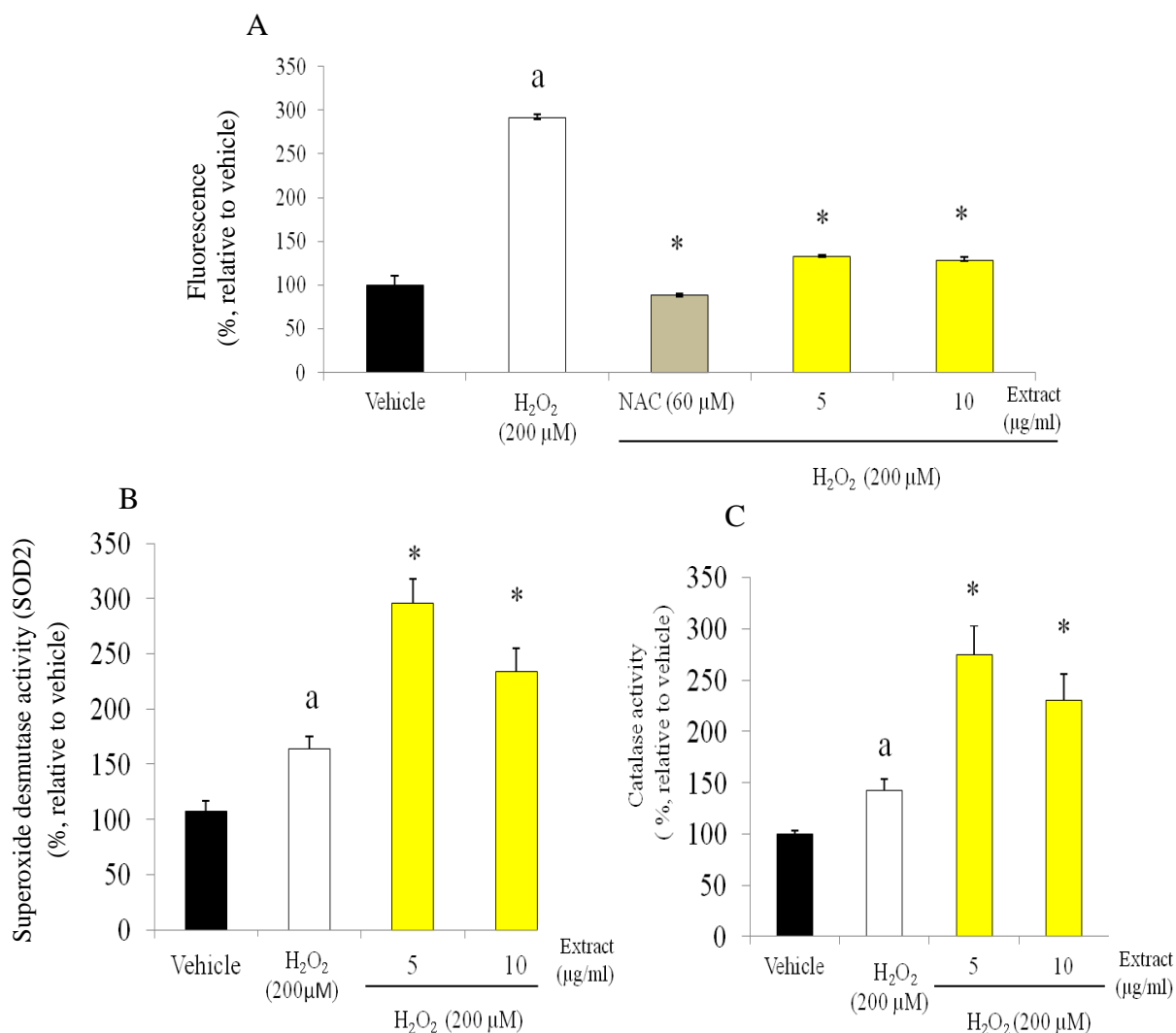
Figure 4.3. Reactive oxygen species scavenging (A) and anti-oxidative enzyme activities (B & C) of SLE against hydrogen peroxide (200 μM) induced reactive oxygen species generation in differentiated hippocampal cells (H19-7). Hippocampal cells (H19-7) were treated with SLE and hydrogen peroxide as described in figure 4.2. Results are expressed relative to vehicle (phosphate buffered saline) control. N-acetyl cystine (NAC) is an anti-oxidant and act as a positive control. 2'-7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) assay was used to quantitate reactive oxygen species concentrations. Each value represents the mean ± SEM, (n=3) obtained from 3 different independent experiments running each sample in duplicate. [a] indicates significant difference from control group (vehicle treated) at $P < 0.05$. [*] indicates significant difference from positive control (hydrogen peroxide treated cells) at $P < 0.05$.

A

Undifferentiated cells   Differentiated cells

                Control   Control   SLE 5   SLE 10

Cytochrome C

Caspase 9

Caspase 3

β actin

B   SLE (μg/ml)
                                  +H₂O₂ (200 μM)

            Control   H₂O₂   5   10

Caspase 3

Cytochrome C

β actin

■ Control (undifferentiated cells)   □ Control (differentiated cells)

□ SLE 5 (undifferentiated cells)   ▨ SLE 10 (differentiated cells)

C
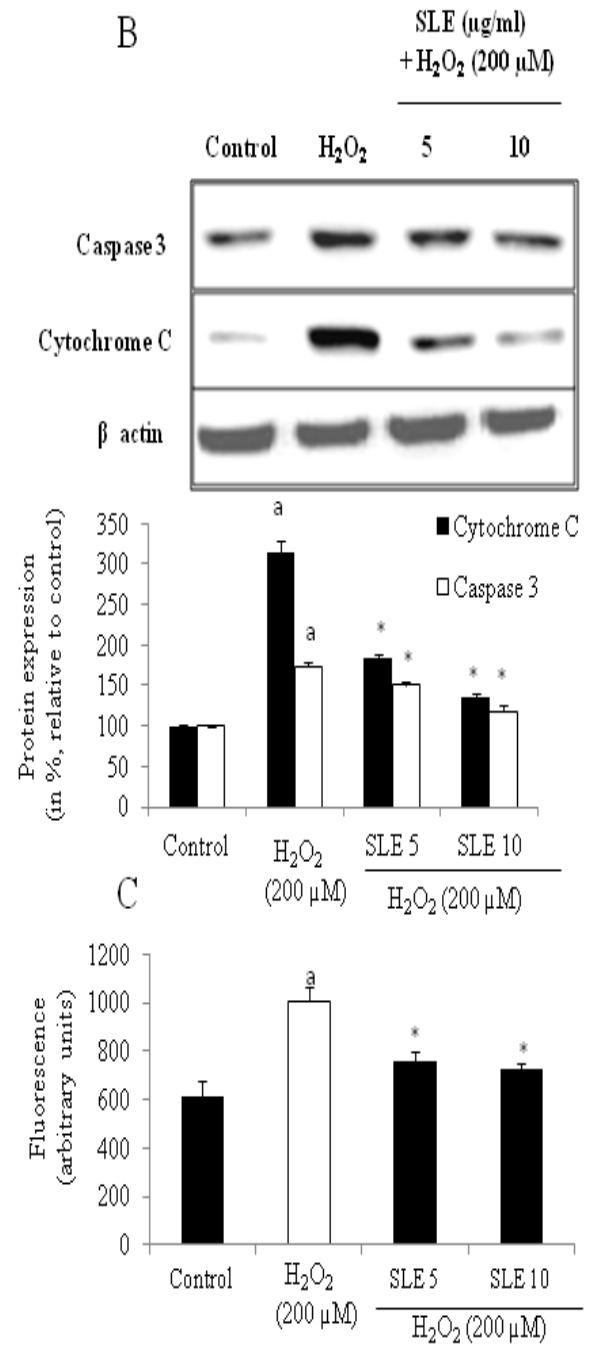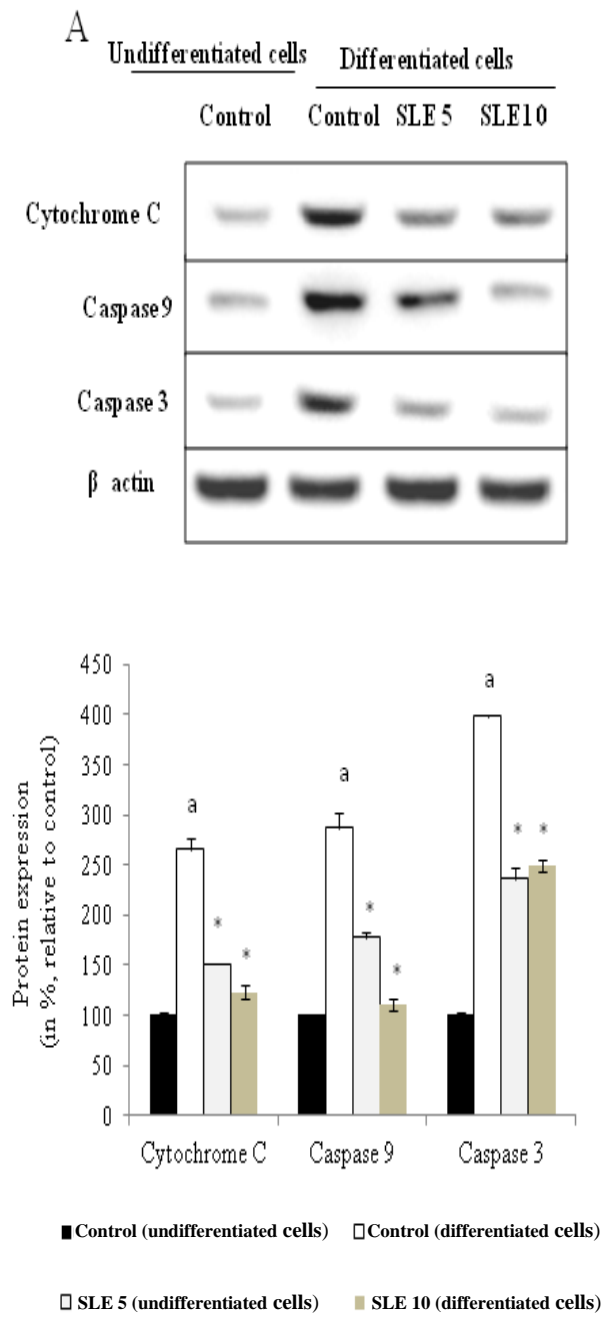
Figure 4.4. Effects of SLE on pro-apoptotic molecules expression in differentiated H19-7 cells treated without (A) or with hydrogen peroxide (200 μM) (B). SLE suppressed caspase-3 enzyme activity induced by hydrogen peroxide (200 μM) treatment in differentiated hippocampal cells by SLE (C). Protein expressions (cytochrome C, caspase 9, cleaved caspase 3) are expressed relative to control (phosphate buffered saline) control after normalized with β actin. Hippocampal cells (H19-7) were treated with SLE and hydrogen peroxide as described in figure1. Each value represents the mean ± SEM, (n=3) obtained from 3 different independent experiments. [a] indicates significant difference from control group (vehicle treated) at $P < 0.05$. [*] indicates significant difference from positive control (hydrogen peroxide treated cells) at $P < 0.05$.
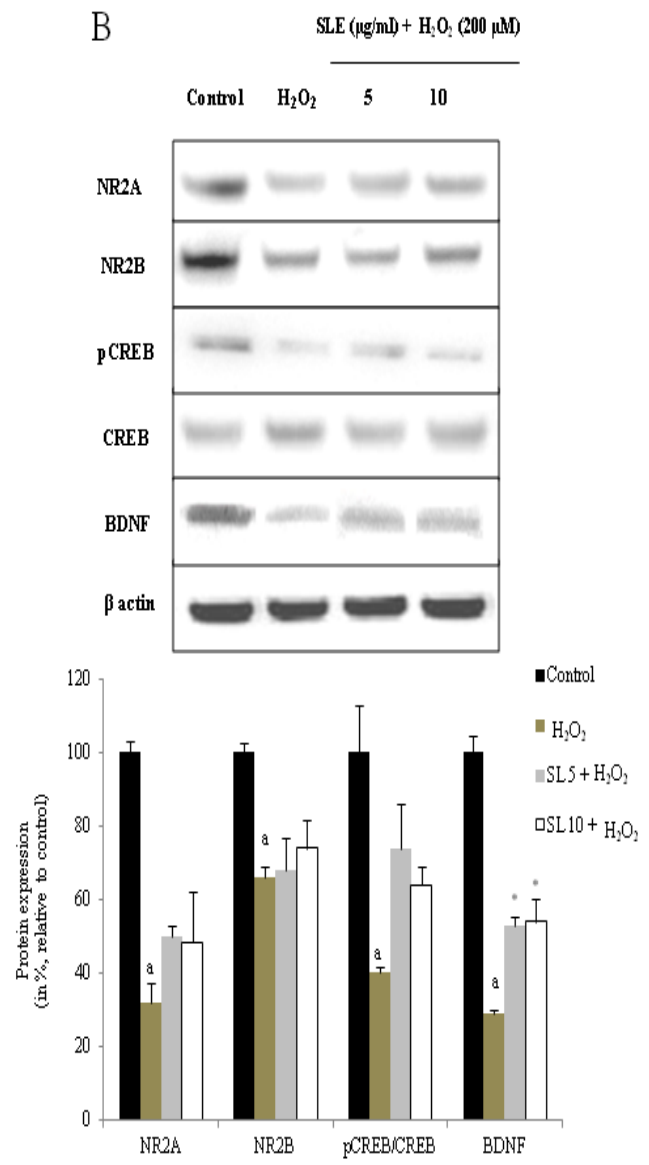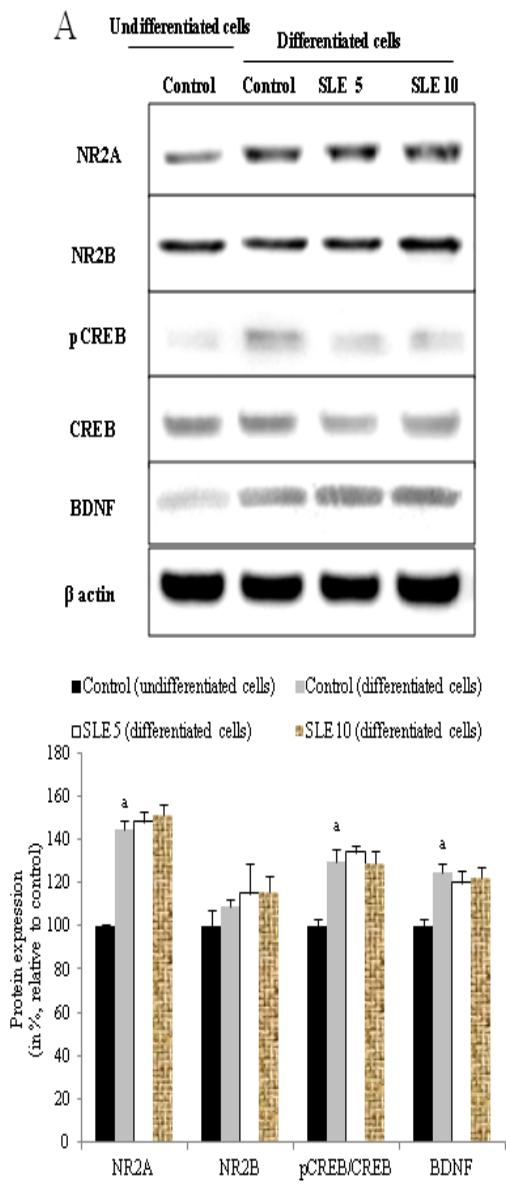
Figure 4.5. Effects of SLE on expression of memory markers (NR2A and NR2B), neurotrophic factors (BDNF) and activated transcription factor (pCREB) in differentiated H19-7 cells treated without (A) or with hydrogen peroxide (200 µM) (B). Protein expressions are expressed relative to control (phosphate buffered saline) control after normalized with β actin. Each value represents the mean ± SEM, (n=3) obtained from 3 different independent experiments. [a]indicates significant difference from control group (vehicle treated) at $P <0.05$. [*]indicates significant difference from positive control (hydrogen peroxide treated cells) at $P <0.05$.

4.5. Discussion

In our previous study we found that oxygen containing nucleophilic t-butyl hydroperoxide-induced lipid oxidation and generation of reactive oxygen species in brain tissue homogenate was inhibited by the ethanolic extract of *S. lateriflora* (SLE). The effectiveness of SLE is attributed to the presence of anti-oxidative flavonoids, polyphenols and glutathione (Lohani et al., 2013b). Hydrogen peroxide is frequently used to induce oxidative stress that can lead to H19-7 and PC12 cells death via apoptosis (Pugazhenthi et al., 2003). In our study we found that SLE protected both H19-7 and PC12 cells against hydrogen peroxide-induced cell death by decreasing reactive oxygen species concentration. Reactive oxygen species scavenging activities of SLE was supported by our previous research findings (Lohani et al., 2013b).

Superoxide dismutase (SOD) and catalase (CAT) are major anti-oxidative enzymes present in a cell that neutralize reactive oxygen species therefore maintain cell homeostasis (Huang et al., 2012; Mian and XiQiang, 2009). In contrast to previous research findings (Zhu et al., 2013), activities of both MnSOD (SOD2) and CAT enzymes in H19-7 cells were increased by hydrogen peroxide treatment. Ethanolic extract of *S. lateriflora* further increased activities of MnSOD and CAT in H19-7 cells treated with hydrogen peroxide which is responsible for eliminating oxygen free radicals and therefore one of the compensatory cellular responses to avoid the development of oxidative stress in H19-7 cells. Enhanced SOD and CAT activities by SLE are attributed to presence of baicalin and wogonin flavonoids (Cheng et al., 2011; Waisundara et al., 2011; Wen et al., 2011; Wu et al., 2011).

Apoptosis is one of the major mechanism by which cell death occurs in brain and oxidative stress is identified as a major contributor for neuronal apoptosis, therefore we examined anti-apoptotic activities of SLE against oxidative stress induced H19-7 cell death. Programmed cell death or apoptosis occurred by two mechanisms in H19-7 cells. The first

was initiated by removal of serum from undifferentiated cells, and the second was a consequence of neuronal differentiation (Eves et al., 1996). Therefore caspase-3, caspase-9 and cytochrome C expression was found to increase in serum deprived differentiated cells compared to serum enriched undifferentiated cells. Caspase-3, caspase-9 and cytochrome C proteins are considered as a marker of apoptosis. Increased apoptosis of differentiated H19-7 cells by hydrogen peroxide treatment is consistent with previously reported findings (Pugazhenthi et al., 2003). However, SLE suppressed expression of these pro-apoptotic markers in serum deprived differentiated H19-7 cells treated with or without hydrogen peroxide compared to only vehicle treated serum deprived differentiated control cells or serum enriched differentiated cells, respectively. Furthermore, SLE suppressed caspase 3 activities in hydrogen peroxide treated differentiated H19-7 cells. Decreased expressions and activities of apoptotic markers by SLE suggest anti-apoptotic activities of *S. lateriflora*. It could be possible that presence of flavanoids in SLE stochiometrically reduced cytochrome C expression in mitochondria (Lagoa et al., 2011). Among flavonoids, baicalin inhibits caspase 3 expression (Cao et al., 2010; Liu et al., 2006) and chrysin suppress cytochrome C release from mitochondria and caspase 3 enzyme activities (Izuta et al., 2008).

The cyclic AMP-responsive element binding protein (CREB) is a post-translationally activated transcription factor that plays an important role in antioxidant capacity of a cell and has been implicated in neuronal growth and survival (Walton et al., 1999). Brain derived neurotrophic factor (BDNF) is critically involved in normal brain physiology, not only as a potential anti-oxidant mediator (Chan et al., 2010), but also in regulation of neurogenesis and neuroplasticity (Lee and Son, 2009). Increased expression of activated CREB and BDNF in differentiated cells compared to undifferentiated cells is consistent with previous research findings (Somoza et al., 2010; Yang et al., 2004). However, SLE did not modulate expression of these neuro-protective proteins expressions in differentiated H19-7 cells. Decreased

expression of activated CREB and BDNF by hydrogen peroxide in differentiated neurons contributed to oxidative stress induced cell death (Chan et al., 2010; Sarvestani et al., 2013). However, SLE increased BDNF expression in differentiated H19-7 cells treated with hydrogen peroxide suggesting other possible mechanism by which SLE exhibits neuroprotective activities. It could be possible that presence of flavonoids wogonin and baicalein in *S. lateriflora* contribute to increased BDNF expression by SLE (Jeon et al., 2010).

Oxidative stress impaired cognitive behavior in animal model (Fukui et al., 2002). Therefore, decreased expression of N-methyl-D-aspartate receptor subunits (NR2A, NR2B) by hydrogen peroxide suggested deteriorated glutamatergic transmission and associated physiological function such as cognition and memory (Soman et al., 2012). However SLE did not ameliorate hydrogen peroxide induced decreased expression of NMDA subunits in H19-7 cells. Increased NR2A subunit expression but not NR2B subunit expression in differentiated cells compared to undifferentiated cells could be due to the switch from predominance of NMDA receptors rich in NR2B subunits to that of NMDA receptors rich in NR2A subunits in differentiated cells (van Zundert et al., 2004).

In summary results of the research findings demonstrate that the SLE has protective effects via suppressing apoptosis and by increasing anti-oxidative capacity of neurons therefore, provide novel insights into the molecular mechanisms underlying the neuroprotective activity of *Sctellaria lateriflora*. Oxidative stress has been associated with the pathogenesis of a range of neuropsychological and neurodegenerative disorders including Alzheimer's and Parkinson's diseases, obsessive–compulsive and panic disorders (Bouayed et al., 2009; Uttara et al., 2009). Therefore, the results indicate a nutraceutical value for the SLE and suggest its potential prophylactic activity against neurological diseases. However,

further studies are required to determine the neuroprotective activities of SLE in animal and

human models.

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5. Anti-inflammatory and immunomodulatory activities of *Scutellaria lateriflora*

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The number of figures: 04; The number of tables: 00

Supplementary online materials: No

Running title: Anti-inflammatory activities of *Scutellaria lateriflora*

In preparation for publication: **Immunopharmacology and Immunotoxicology Journal**

Abstract

Pro-inflammatory cytokines stimulate both innate and adaptive mechanisms by activating neutrophils, macrophages and lymphocytes. However, excessive inflammation adversely affects overall health. *Scutellaria lateriflora* is a native plant of North America and contains higher concentrations of chemically active flavonoids than other commonly used species of the plant of genus *Scutellaria*. To date very limited research studies have been done to measure the pharmacological activities of *S. lateriflora*. The purpose of this study was to determine the immunomodulatory and anti-inflammatory properties of *S. lateriflora*. Experiments were designed to measure the effects of an alcoholic extract of *S. lateriflora* (SLE) on inflammatory cyclooxygenase-1(COX-1), cyclooxygenase-2 (COX-2) and 15-LOX (15 Lipoxygenases) enzyme activities, and immunomodulating T lymphocytes activation and major histocompatibility complex class-II (MHC-II) expression in bovine monocyte-derived dendritic cells (MoDCs). The SLE inhibited inflammatory COX-1, COX-2 and 15 LOX activities but did not affect T cell proliferation, MHC-II expression on MoDCs and pro-inflammatory cytokines production by T cells. Our results indicate anti-inflammatory activities of SLE and therefore is a potential treatment for various inflammation driven diseases.

5.1. Introduction

In general, inflammation is considered as a protective innate immune mechanism that is mediated by pro-inflammatory cytokines (TNF-α, IL-1 and IL-6) and are released from innate immune cells (Janeway et al., 2001). These pro-inflammatory cytokines stimulate innate and adaptive immune cells, including neutrophils, macrophages, T and B lymphocytes. Inflammation stimulates production of reactive oxygen radicals that act to kill pathogens (Janeway et al., 2001). Prostaglandins and leukotrienes are important mediators of inflammation and are responsible for cellular changes such as cell membrane permeability (Ricciotti and FitzGerald, 2011). However, excessive or chronic inflammation adversely affect overall health. For example, inflammation in gut epithelium can lead to enteritis (MacDonald and Monteleone, 2005). Similarly, inflammation contributes to the pathogenesis of neuropsychological and neurodegenerative disorders (Amor et al., 2010; Andrews et al., 1987; Graff et al., 2009) . Currently used pharmaceutical agents including non-steroidal anti-inflammatory drugs (NSAIDs) reduce inflammation via suppression of cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) isoenzymes in a non-selective manner (Bombardier et al., 2000; Silverstein et al., 2000). Cyclooxygenase-2 is abundantly expressed in innate immune cells such as neurtrophils, whereas COX-1 is constitutively expressed in most body cells, including the gastric mucosa (Ricciotti and FitzGerald, 2011). Consequently, therapy intended to reduce inflammation have side effects such as suppression of mucous production in gut epithelium and resultant gastric ulcers (Roth, 1996; Wallace et al., 2000) . Recent studies indicated that flavonoids utilize multiple mechanisms to reduce inflammation without causing side effects. They inhibit nitrite production, prostaglandin synthesis and most importantly, selectively suppress COX-2 enzymes without increasing the risk of side effects, including gastric ulcers (Wakabayashi and Yasui, 2000). Synthetic COX-2 inhibitors exhibit anti-inflammatory action with a lower tendency of producing gastric ulcer. Long term dosing

trials have shown that certain COX-2 inhibitors, e.g. rofecoxib, can lead to increased cardiovascular morbidity (Mukherjee et al., 2001).

There is evidence that plants of genus *Scutellaria* might not exhibit side effects on the cardiovascular system, because this herb has a variety of active compounds that modulate inflammation via a combination of different mechanisms.

American skullcap (*S. lateriflora*) and Chinese skullcap (*S. baicalensis*) are the only officially recognized sources for herbal products of genus *Scutellaria* (Zhang et al., 2009). *S. baicalensis* is the most studied species of genus *Scutellaria* (Zheljazkov et al., 2007). All major flavonoids extracted and purified from *S. baicalensis* exhibited significant anti-inflammatory and immunomodulatory activities (Bochořáková et al., 2003; Huang et al., 2006; Kwon et al., 2009). However, *S. baicalensis* is not a native plant of the United States and is grown mainly in China. Most of the cultivated plant materials used in the US for traditional herbal Chinese medicine, including *S. baicalensis*, are imported from China (Craker and Giblette, 2002). *S. lateriflora* is a native plant of North America and contains chemically similar immunomodulatory flavonoids than are found in *S. baicalensis* (Makino et al., 2008; Wakabayashi and Yasui, 2000). Despite the fact that the aerial parts of *S. lateriflora* contains higher concentrations of flavonoids than those from *S. baicalensis* (Makino et al., 2008), very limited studies have examined the pharmacological activities of *S. lateriflora*. The purpose of this study was to examine the immunomodulatory and anti-inflammatory properties of *S. lateriflora*. Our experiments measured the effects of an alcoholic extract of *S. lateriflora* (SLE) on cyclooxygenase-1 (COX-1), cyclooxygenase-2 (COX-2) and 15-Lipoxygenases (15-LOX) enzyme activities, T lymphocyte proliferation and on major histocompatibility complex- II (MHC-II) expression of bovine monocyte derived dendritic cells (MoDCs).

5.2. Materials and Methods

Animals: Three brown Swiss calves (8-12 months of age) housed at the Dairy Farm, South Dakota State University (Brookings, SD, U.S.A.) were used in this study. All animals were examined and found to be healthy. Animal handling and blood collection was done as per the guidelines of South Dakota State University Institutional Animal Care and Use Committee.

Plant material and chemicals: S. lateriflora seeds were obtained from Horizon Herbs LLC (Lot no. 4232, William, OR) and sown in Marvyn loamy sand at the Horticulture Unit of the E.V. Smith Research Center (Alabama Agriculture Experiment Station, Shorter, Alabama). The seeds were sown in the spring of 2007 and the above ground portions of each plant were cut 7.5 cm from the ground using pruning shears during the following summer. Collected aerial parts of plants were placed in open paper bags and dried in a forced-air dryer (Model AA-5460A, Parameter Generation and Control Inc, Black Mountain, NC) at 40 ^{o}C for 3 days. The dried aerial parts of plants were ground to a fine powder using a Wiley Mill (Model 4, Thomas Scientific, Swedesboro, NJ) mill and stored at room temperature in large cloth bags that allowed movement of air. All chemicals, except where noted, were purchased from Sigma-Aldrich (St Louis, MO).

Preparation of alcoholic extracts of S. lateriflora. The highest concentration of plant flavonoids was extracted in 70% ethanol as compared to other solvents (Lohani et al., 2013b). Alcoholic extracts of *S. lateriflora* (SLE) was used to determine the mechanisms of neuroprotective activities. Ground, dried powder of *S. lateriflora* leaves and stems was either mixed in 70% aqueous ethanol or distilled water for 24 hours at room temperature on a platform shaker (Model VXR S10, Tekmar technology, Funkentstort, West Germany). The samples were centrifuged for 30 min at 1500 rpm and the supernatant (alcoholic extract) collected, filtered through 0.2 µm filter paper, evaporated and dried. Dried extract was stored at -4 ^{o}C until further use.

Effect of SLE on Cyclooxygenase-1(COX-1) and cyclooxygenase-2 (COX-2) activities:
Cyclooxygenase-1(COX-1) and COX-2 inhibitory activities of the alcoholic extract of *S. lateriflora* (SLE) was measured using a calorimetric COX (ovine) screening assay kit (Cayman Chemical Company, Ann Arbor, MI, U.S.A). The inhibitory activities of SLE were measured by monitoring the N,N,N, N tetramethyl-p-phenylenediamine (TMPD) at 590 nm, followed by incubation of either ovine COX-1 or COX-2 combined with arachidonic acid and heme. The enzyme mixtures were incubated for 5 minutes at 25 °C with SLE prior to addition of arachidonic acid (final concentration 1.1 mM) and TMPD. TMPD undergoes oxidation producing a highly colored product. Inhibitory activity on COX was calculated using the following equation:

$$I \% = [(A_{blank} - A_{sample}) / A_{blank}] \times 100$$

where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} is the absorbance of the test compound. The extract concentration resulting in 50 % inhibition (IC_{50}) was calculated from a graph plotting inhibition percentage against extract concentration.

Effect of SLE on 15-Lipoxygenase activity: The 15-lipoxygenase (15-LOX) inhibitory activity of ethanolic extract was examined using a Lipoxygenase Inhibitor Screening Assay Kit (Cayman Chemical, Ann Arbor, MI). Linolenic acid was used as a substrate for soy 15 LOX enzyme. The assay detects the formation of hydroperoxides by measuring activity of 15 LOX on linolenic acid conversion to chromogen. The kit consists of FeSO4.7H2O and NH4SCN, which convert to bright yellow in the presence of 15-LOX. The yellow color (chromogen) was read at 490 nm. Inhibitory activity on 15 LOX was calculated using the equation as described earlier on COX.

Culture of macrophages and SLE treatment in order to study production of pro-inflammatory cytokines interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α): RAW 264.7

macrophage cells were grown in Roswell Park Memorial Institute (RPMI) 1640 medium, supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin and 100 units/ml streptomycin sulfate. The cells were incubated in a humidified 5 % CO_2 atmosphere at 37 °C. These cells were pretreated with a range of concentrations of SLE (0-100 μg/mL) or dexamethasone (positive control) for 60 minutes. After 60 minutes, medium was replaced with fresh RPMI 1640 medium and lipopolysaccharides (LPS, 200 ng/mL) were added for 24 hours to stimulate Raw 264.7 macrophage cells. After 24 hours, macrophage cells were collected from the flask and homogenized for 30 seconds in 0.1 M phosphate buffer saline (PBS, pH 7.4). The homogenates were centrifuged for 10 minutes at 17,000 Xg at 4°C, and the supernatant was used for determination of TNF-α and IL-6.

Isolation of dendritic cells and SLE treatment in order to study production of interferon gamma (IFN-γ): Gradient centrifugation method was used to isolate Peripheral Blood Mononuclear Cells (PBMCs) from blood of unvaccinated calves as described by (Rajput et al., 2014). PBMCs were grown in six-well plates containing Roswell Park Memorial Institute medium (RPMI) 1640 medium supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37 °C for three hours. The adherent cells were separated out from 6 well plates using proteolytic and collagenolytic enzyme Accutase (eBioscience, San Diego, CA, USA). Monocytes were confirmed as positive for Major Histocompatibility Complex Class-I (MHC-I^{+}) and class-II (MHC-II^{+}), and Cluster of Differentiation 14 (CD14^{+}) cell surface markers and purity of the cells was determined to be 98.24 % by Flow cytometry (Becton-Dickson, Mountain View, CA, USA). Isolated monocytes were differentiated into monocyte-derived dendritic cells (MoDCs) using RPMI 1640 medium supplemented with 10% FBS, 1mM sodium pyruvate, streptopenicillin (100 U/ml), bovine recombinant Granulocyte Monocyte Colony Stimulating Factor (GMCSF) (100 ng/ml) and interleukin-4 (IL-4) (200 ng/ml). Monocyte-derived dendritic cells (MoDCs) were confirmed

morphologically and phenotypically through MHC-I$^+$, MHC-II^{++}, CD86$^+$, CD14$^-$, CD21$^+$ and CD 205$^+$ antibody staining cell surface staining (VMRD Inc, Pullman, WA, USA). MoDCs were treated with SLE at concentration of 50 µg/ml, 100 µg/ml, 250 µg/ml and 500 µg/ml for 48 hrs. After 48 hrs, MoDCs were washed with PBS and stained with anti-mouse MHCII antibody. Bound mAb was detected with fluorescein isothiocyanate (FITC) - labelled anti-mouse IgG (Southern Biotechnologies). The cells were fixed with 1 % formaldehyde and assessed for MHCII expression by Fluorescence Activated Cell Sorting (FACS Calibur) flow cytometer (Becton-Dickson, Mountain View, CA, USA).

ELISA for IL-6, TNF-α and IFN-γ: Standard protocol for IL-6 and TNF-α ELISA was followed according to the manufacturer's instructions (Quantikine M rat IL-6 and TNF-α immunoassay kit; R&D Systems, Minneapolis, MN) to measure the levels of IL-6 and TNF-α cytokines in supernatant of cultured Raw 264.7 macrophage homogenate exposed to SLE and /or LPS. Briefly, 50 µL of assay dilutents and IL-6 and TNF-α standards or supernatants were added to wells of microplate strips coated with anti mouse IL-6 or TNF- α antibodies. These microplate strips were incubated at room temperature for 2 hrs. After 2 hrs, each well was aspirated and washed 5 times with buffer. After washing, 100 µl of conjugate were added to each well and the microplate strips were incubated at room temperature for an additional 2 hrs. After 2 hr of incubation, each well was washed 5 times and 100 µl substrate solution was added and incubated at room temperature for 30 minutes. Finally, 100 µL of stop solution to terminate the reaction was added to each well and within 30 minutes the microplate strips were read at 450 nm. All samples were run in duplicate. Blanks (no macrophage supernatant) and serial dilutions of mouse IL-6 or TNF- α standards were present on each plate. Samples with absorbance values that exceeded the upper limit of the absorbance of standards and duplicate samples with absorbance values that varied more than 10% were run again. The optical density of each sample (in duplicate) was determined with a microplate

spectrophotometer (Benchmark Plus; Bio-Rad, Hercules, CA). IL-6 and TNF-α content were calculated according to the manufacturer's instructions and reported as picograms per milligram protein. The assay was repeated, and the averages of the two runs were reported. The IFN-γ concentration was detected by Bovine IFN Gamma Kit (Thermo scientific, Rockford, IL) according to manufacturer's recommendation.

T cell isolation and SLE treatment: Calves (8-12 months of age) were vaccinated with live bovine viral diarrhea vaccine and blood was collected 7 d after vaccination. T cells were isolated from PBMCs and treated with SLE extract at concentration of 50 μg/ml, 100 μg/ml, 250 μg/ml and 500 μg/ml. The viability of untreated (PBS treated control) and treated T cells was measured by trypan blue exclusion assay as previously described (Strober, 2001). The T cell mitotic activities of SLE was measured by Carboxyfluorescein Succinimidyl Ester (CFSE) proliferation assay (Invitrogen, Carlsbad, CA) as described by Kloosterboer et al., (2006) with slight modification (Kloosterboer et al., 2006). Briefly, T cells were suspended in 4 ml PBS to achieve a final concentration of $1x \ 10^6$ cell/ ml. Four microliters of 50 mM CFSE was added to the T cell suspension. T cells were stained with CFSE for 8 minutes at 37 ^{o}C. The reaction was stopped by adding ice cold fetal bovine serum (FBS). T cells were washed 2 times with 10% FBS in PBS and suspended in RPMI 1640 medium supplemented with 10% FBS, 1mM sodium pyruvate and streptopenicillin (100U/ml), with or without (control) SLE. T cells without CFSE were used as a negative control. T cells were incubated at 37 ^{o}C for 5 days. T cell proliferation was measured by Flowcytometry using the FL1 channel.

5.3. Results

Pro-inflammatory enzymes activitiy was repressed by SLE: The SLE was analyzed for COX and LOX inhibitory activities using COX and LOX inhibitor screening assay kits, respectively. SLE significantly inhibited COX-1 and COX-2 activities compared to vehicle

(PBS) control ($p<0.001$). The IC$_{50}$ values for COX-1 and COX-2 were calculated to be 50 μg/mL and 125 μg/mL, respectively (Figure 5.1 A and 5.1 B). Only the highest concentration (1000 μg/ml) of SLE inhibited 15-LOX activity by 10 ± 1.5% when compared to vehicle control ($p<0.05$) (Figure 5.1 C).

Cytotoxicity of SLE for RAW 264.7 macrophage, myeloid dendritic cells and T cells: To evaluate cytotoxicity of SLE in immune cells, cell viability was determined using an MTT assay. The viability of the RAW 264.7 macrophage cells and dendritic cells was not altered by concentrations of SLE up to concentration of 500 μg/mL (Figures 5.2 A and 5.3 A). However, SLE reduced viability of T cells at 250 μg/mL and 500 μg/mL concentrations (Figure 5.3 B).

The SLE effect on lipopolysaccharides (LPS)-stimulated cytokines production in RAW 264.7 macrophage: The effect of SLE on TNF-α and IL-6 cytokine productions by lipopolysaccharides (LPS) -stimulated RAW 264.7 macrophage cells was measured with ELISA as described previously. After 30 minutes of exposure to a range of SLE concentrations RAW 264.7 macrophage cells were stimulated with LPS and the levels of TNF-α and IL-6 cytokine produced were measured by analyzing the homogenate of cultured cells supernatant. As shown in Fig. 5.2 B and C, SLE did not inhibit either TNF-α or IL-6 cytokine production. LPS (the positive control) stimulated both TNF-α and IL-6 cytokine production by RAW 264.7 macrophage compared to vehicle control demonstrating the normal response of these macrophages. Dexamethasone, another positive control, significantly suppressed LPS-stimulated TNF-α and IL-6 cytokines productions by RAW 264.7 macrophage (Figure 5.2 B and 5.2 C), but SLE did not affect production of these cytokines even at 100 μg/ml.

SLE modulatation of MHC-II expression in myeloid derived dendritic cells (MoDCs): The recovered myeloid derived dendritic cells (MoDCs) were characteristically 5-7 times larger

than monocytes with long dendrites (Rajput et al., 2014). Flowcytometry analysis of their surface markers confirmed induction of MoDCs. Recovered MoDCs were positive for major histocompatibility complex (MHC-I$^+$, MHC-II$^+$), cluster of differentiation 86 and 205 (CD86$^+$ and CD205$^+$) and negative for CD14$^-$ and CD21$^-$ surface molecules. MHC-II expression in MoDCs was not significantly modulated when treated with non-toxic concentrations (50 - 500 µg/ml) of SLE for 48 hrs compared to vehicle (PBS) control (Figure 5.3 C). Treatment with 100 µg/ml SLE increased MCH-II expression about 30 % but was not significantly different due the large variation (Figure 5.3 C).

The SLE does not stimulate proliferation of T cells and IFN-γ production: To investigate the mitogenic effects of SLE on T cells, CFSE (carboxyfluorescein succinimidyl ester) proliferation assay were conducted and ELISA was performed to determine affect of SLE treatment on IFN-γ production by T cells. T cells were treated with different concentrations of SLE for 48 hrs. SLE neither induced T cell proliferation nor modulated production of IFN-γ by T cells (Figure 5.4 A and 5.4 B). However, the known mitogen concavalin A significantly increased T cells proliferation compared to vehicle control (Figure 5.4 A). Treatment with 100 µg/ml SLE was not significantly different compared to vehicle controls (Figure 5.4 A).

5.4. Figures and Legends

A

B

C
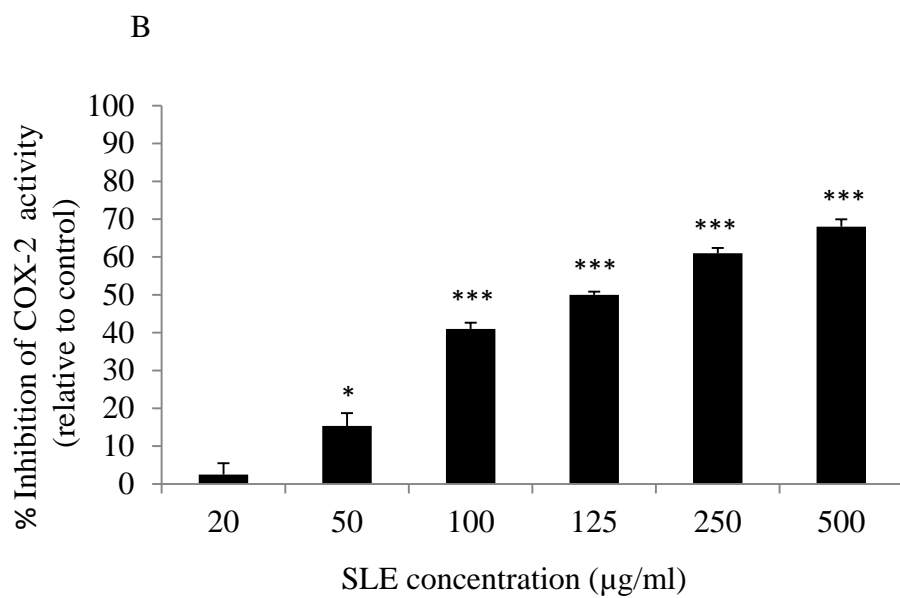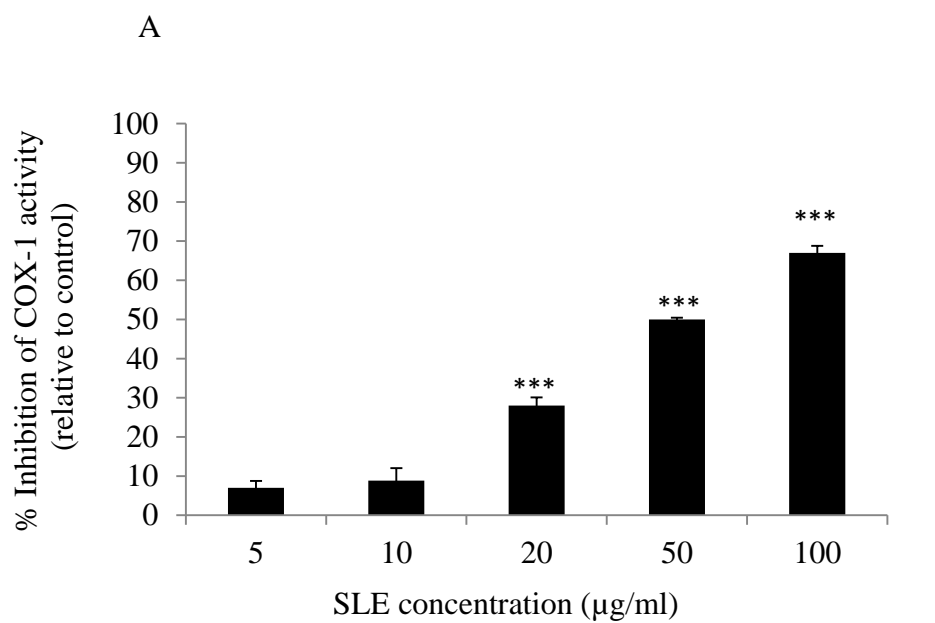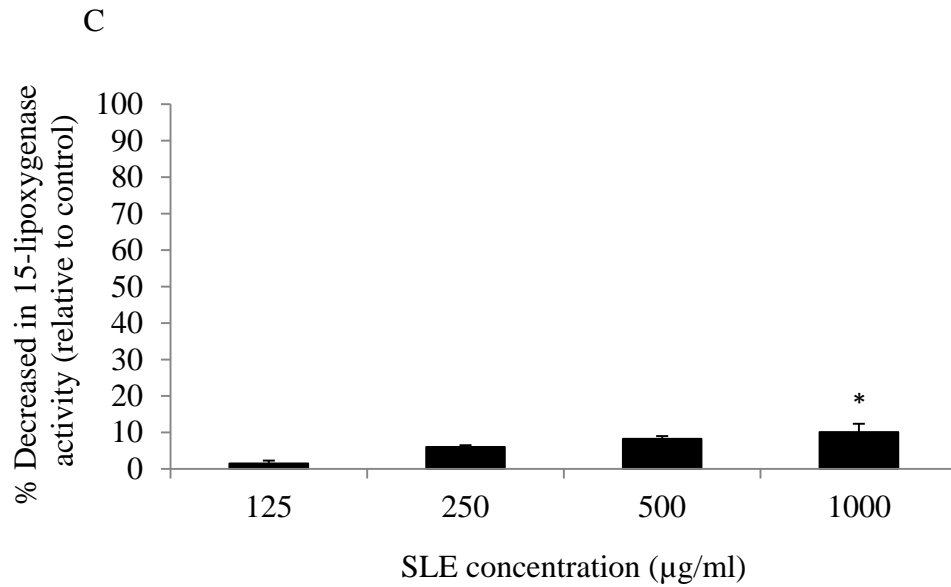
Figure 5.1. Changes in anti-inflammatory activities of ethanol extracts of *S. lateriflora* (SLE) in mixture contained arachidonic acid (for COX-1 and COX-2) or linoleic acid (for LOX 15) substrate. Results are expressed as percentage decrease in absorbance at 590 nm (for COX-1 and COX-2) or 490 nm (for 15 LOX), with respect to vehicle control (PBS treated group). Each value represents the mean ± SEM, (n=6). Significantly different from the vehicle (PBS) treated control group *p <0.05 and ***p<0.001.

A

B

94

C
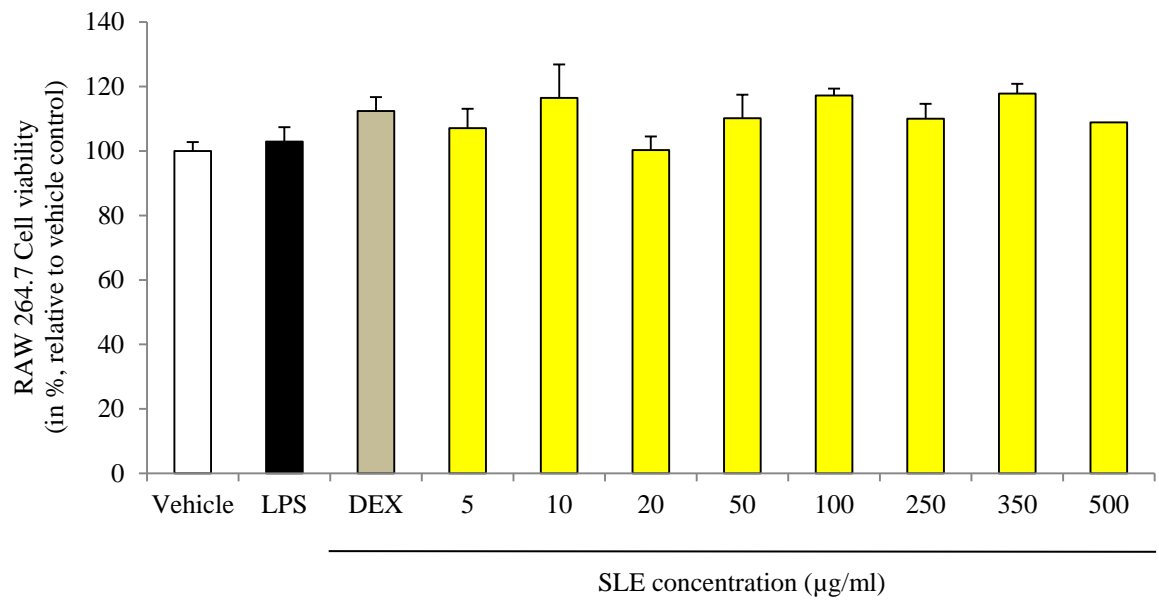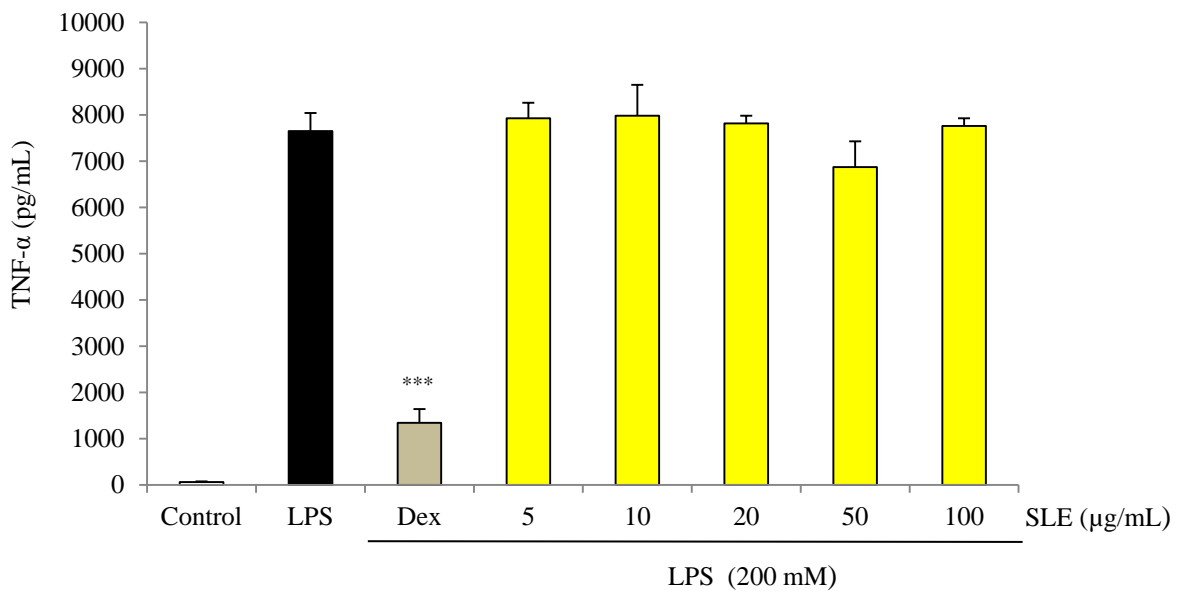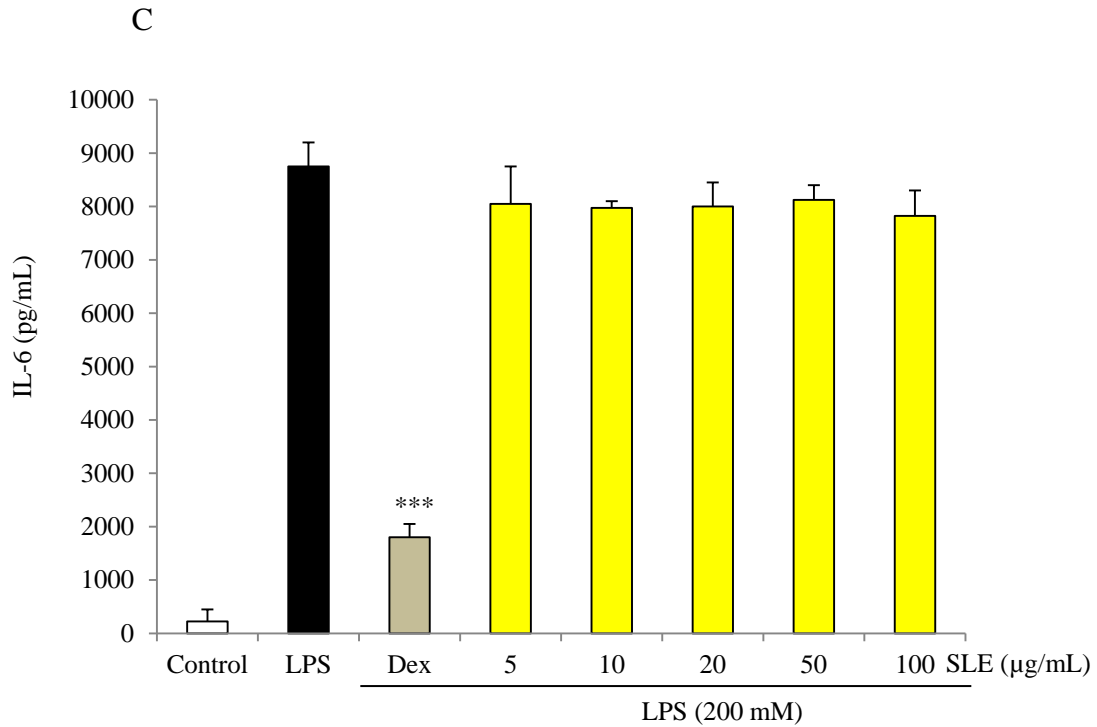
Figure 5.2. The MTT assay was used to evaluate the cytotoxicity of SLE to macrophage (RAW 264.7) cells after exposure to a range of SLE concentration (A). Effects of SLE on lipopolyssachrides (LPS)-induced TNF-α (B) or IL-6 (C) production by RAW 264.7 macrophage cells was not significant. Pretreatment of macrophage (RAW 264.7) cells with SLE (5, 10, 20, 50 and 100 μg/mL) for 60 min prior to incubation with LPS (200 ng/mL) for 24 h. Production of IL-6 and TNF- α were measured by ELISA. Significantly different from the LPS treated control group *p <0.05 and ***p<0.001.

A

B

C
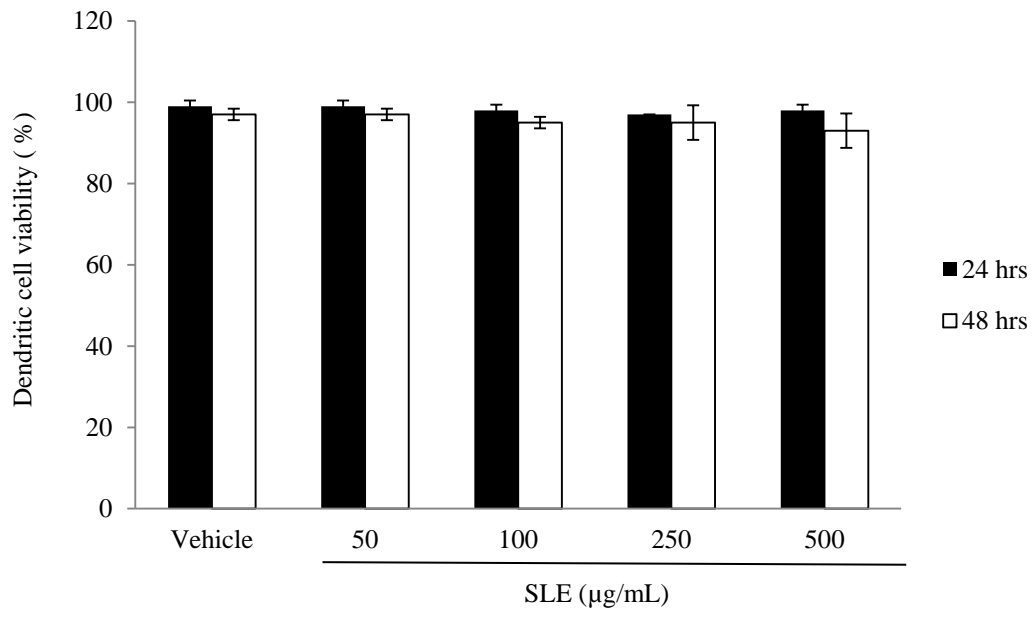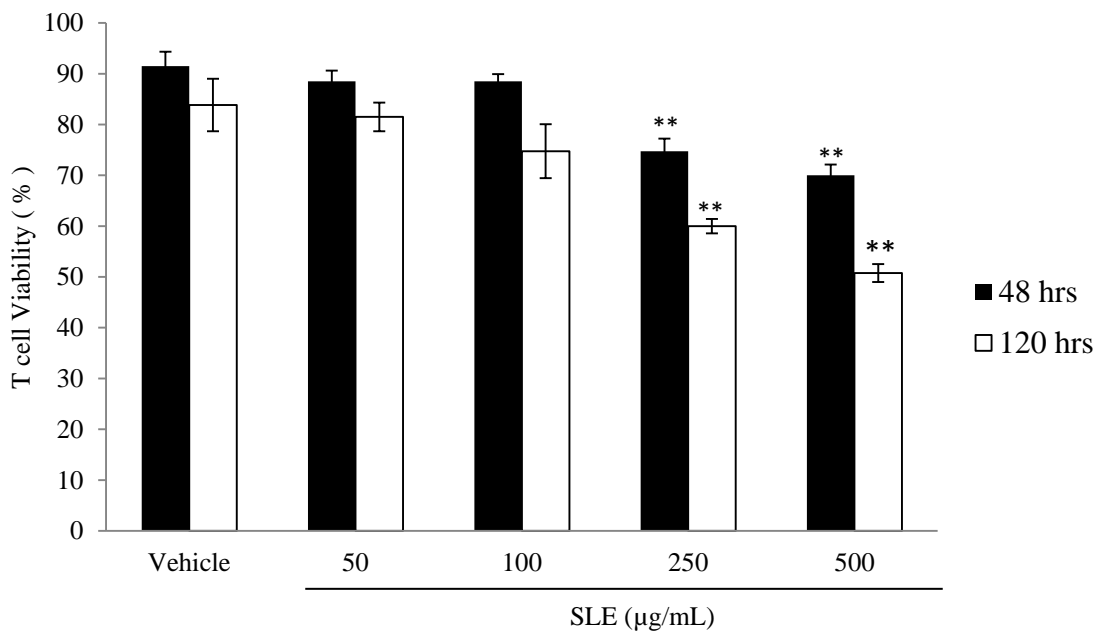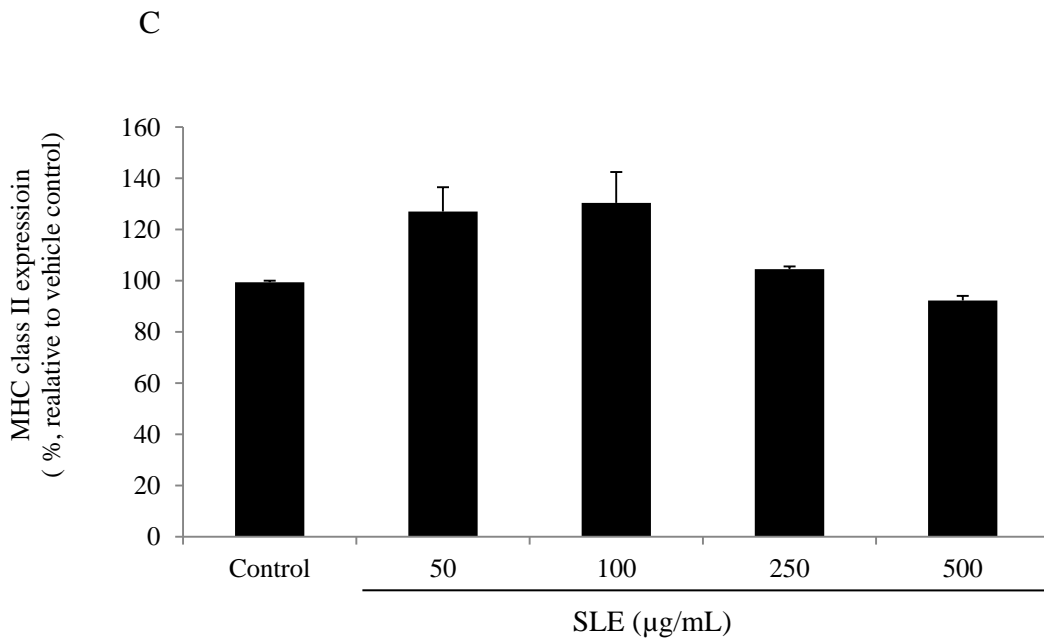
Figure 5.3. The cytotoxicity of SLE on dendritic cells (A) and T cells (B) at the range of concentrations was evaluated by an MTT assay. Dentritic cells were incubated for 24 hrs or 48 hrs with SLE, T cells were incubated for 48 hrs or 120 hrs with SLE. Isolated monocytes were differentiated into monocyte-derived dendritic cells (MoDCs) by culturing with using bovine recombinant GMCSF (100ng/ml) and IL-4 (200ng/ml). Differentiated MoDCs were confirmed morphologically and phenotypically through expression of MHCI, MHCII, CD86, CD14, CD21 and CD 205 by Flowcytometry. Dendritic cells were incubated with nontoxic concentrations of SLE for 48 hrs and analyzed for MHC-II expressions (C). Significantly different from the control (vehicle treated) group at **$p<0.005$.
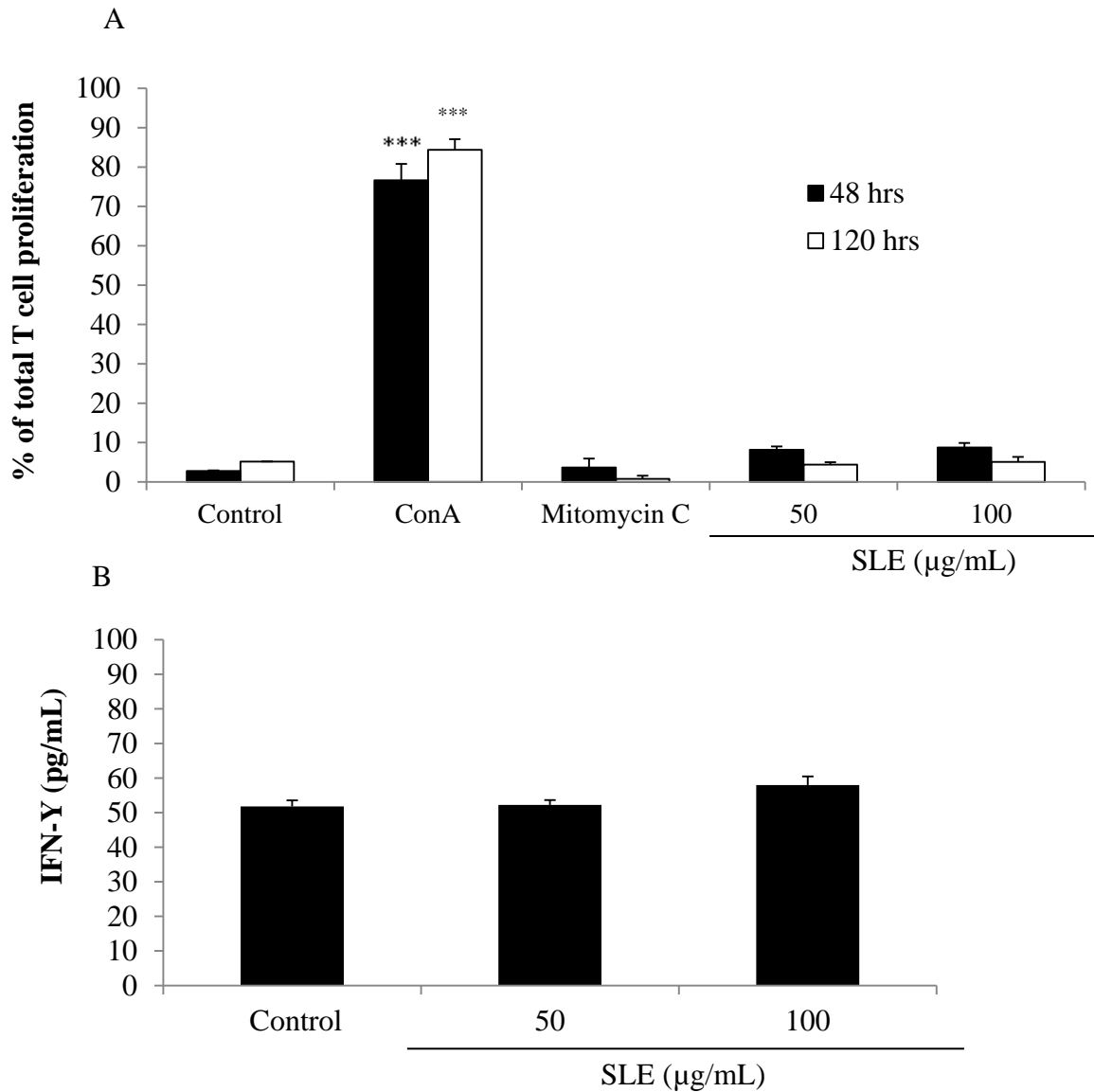
Figure 5.4. T cells proliferation (A) and IFN-γ production (B) were evaluated by CFSE (carboxyfluorescein succinimidyl ester) assay and ELISA, respectively. A) T cells were incubated with a range of concentrations of SLE for 48 hrs or 120 hrs. Concavaline A (ConA) was used as a positive control because it is known to induce proliferation of T-cells and mitomycin C was used as a negative control because it inhibits T cells proliferation. B) Culture supernatants were collected after 48 hrs and analyzed for IFN-γ levels. Significantly different from the control (vehicle treated) group at ***$p<0.001$.

5.5. Discussion

In this study, we investigated the potential anti-inflammatory and immunomodulatory effects of flavonoid-rich ethanol extract of the American herb *S. lateriflora*. We showed that SLE has strong anti-inflammatory activities. Until now no reports examining the anti-inflammatory potential of *S. lateriflora* were available. Thus, the present study is the first to analyze the anti-inflammatory effects of *S. lateriflora*. In our study we found that SLE inhibited COX-1 and COX-2 enzyme activities. SLE also exhibited weak inhibitory effects on 15-LOX enzyme activities. Anti-inflammatory activities of SLE could be attributed to the presence of wogonin, baicalein and baicalin flavonoids that suppress either expression or activity of pro-inflammatory mediators (Li et al., 2009). Baicalin, baicalein and wogonin directly attenuate prostaglandin synthesis via inhibiting cyclooxygenase-2 (COX-2) expression or activity (Chi et al., 2003; Gurung and Kim, 2009; Lee and Kim, 2010; Wakabayashi and Yasui, 2000). In addition to COX-2, baicalin inhibits reported to have COX-1 activity (Lee and Kim, 2010). Similar flavonoids (baicalin, baicalein and wogonin) also inhibit 15-lipoxygenase activities (Butenko et al., 1993; Cui et al., 2010; Li et al., 2012c; Song et al., 2013). Cyclooxygenase and lipoxygenase are responsible for generating inflammatory mediators that contribute to pathogenesis of a range of diseases. Therefore, *S. lateriflora,* could be potential therapeutic for inflammatory diseases or disorders.

Lipopolysaccharide treatment increased pro-inflammatory cytokines TNF- α and IL-6 production by macrophages, which is consistent with previous research findings (Oh et al., 2012). However, flavonoid rich ethanol extract of *S. lateriflora* did not suppress LPS-induced pro-inflammatory cytokine production. Severity of inflammation depends on the balance between concentrations of pro- and anti- inflammatory cytokines. Therefore, further research will be needed to explore the effects of *S. lateriflora* on anti-inflammatory cytokine production by innate immune cells. This is supported by the fact that flavonoids present in *S.*

lateriflora induce production of anti-inflammatory cytokines. For example, transforming growth factor-β1 is a major anti-inflammatory cytokine whose gene expression in mouse macrophage cells is up-regulated by baicalin, chrysin and baicalein of *S. lateriflora* in a concentration dependent manner (Chuang et al., 2005; Pan and Halper, 2003) and thus could reduce systemic and localized inflammatory responses. In addition to anti-inflammatory activities, flavonoids present in *S. lateriflora*, either in pure form or extracted from other plants of genus *Scutellaria,* modulate immune function. The cytokine IFN-γ, produced by T cells and NK cells is essential for Th1-type immunity induced against viral and intracellular bacterial infections. In this study the effects of SLE on T cells proliferation and IFN-γ production by these cells was determined. In contrast to previous research, that reported plant extract containing baicalein and wogonin to stimulate both IFN-γ production (Blach-Olszewska et al., 2008; Lim, 2004) and lymphocytes proliferation(Ohtake et al., 2005), results indicate that SLE neither stimulates T cells proliferation nor modulates IFN-γ secretion by T cells. Dendritic cells (DCs) play a crucial role in antigen processing and presentation. DCs are potent activator of naive T cell by producing pro- and/or anti-inflammatory cytokines (Hope et al., 2003; Howard et al., 2004; Iyonaga et al., 2002) along with expression of co-stimulator molecules. MHC-II molecules are constitutively expressed on DCs. However, SLE did not modulate MHC-II expression on DCs compared to controls.

In conclusion our results indicate anti-inflammatory activities of *S. lateriflora* by inhibiting enzyme activities of COX-1 and COX-2, therefore is a potential candidate to treat various inflammatory diseases.

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6. Effects of flavonoids-rich *Scutellaria lateriflora* on synaptic plasticity

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The number of figures: 05; The number of tables: 00

Supplementary online materials: No

Running title: Effects of *Scutellaria lateriflora* on synaptic plasticity

Author disclosures: M. Lohani, M A Buabeid, D Shannon, V Suppiramaniam, D Schwartz, B W Kemppainen and M Dhanasekaran no conflicts of interest.

In preparation for publication: Nutritional Neuroscience Journal

Abstract

Anxiety is one of the most prevalent neuropsychological disorders around the world. Current anxiolytic therapies have severe adverse effects including anterograde amnesia and memory disruption. *Scutellaria lateriflora* has been reported to possess dose dependent anxiolytic activities in a double blind, placebo-controlled study of healthy human subjects. The inhibitory neurotransmitter, gamma amino butyric acid (GABA) is a major amino acid present in *S. lateriflora*. No study has been done to examine effects of *S. lateriflora* on memory function. Therefore, the objective of this study is to investigate the nootropic (memory enhancer) effects of *S. lateriflora*. Alcoholic extracts of *S. lateriflora* (SLE) was prepared from aerial parts of the plant. Synaptic plasticity (strengthen or weaken a synapse) was determined using electrophysiological methods in term of long term potentiation by exposing hippocampal slices with SLE. Single-channel electrophysiological activities of synaptic alpha amino-3-hydroxy-**5-**methylisoxazole-4-propionic acid receptors (AMPAR), N-methyl-D-aspartate receptors (NMDAR) and gamma amino butyric acid receptors (GABAR) were measured in synaptosomes (an isolated synaptic terminal portion from a nerve cell) reconstituted in a lipid bilayer. Protein expression of AMPAR and NMDAR and GABAR receptors was measured after 24 h of SLE exposure in differentiated hippocampal (H19-7) derived cell line. Results showed that SLE decreased long term potentiation (LTP) compared to control. The electrophysiological kinetics of the GABAR, AMPAR and NMDAR channels were enhanced after exposing SLE to synaptosomes compared to control. SLE did not modulate protein expression of AMPAR, NMDAR and GABAR channels compared to control. Increased GABA and glutamate receptors (AMPAR and NMDAR) activities by SLE are attributed to presence of GABA and glutamate, respectively. In conclusion GABA and flavonoids in alcoholic extract of *S. lateriflora* decreased synaptic plasticity by increasing activities of GABA receptors.

6.1. Introduction

Benzodiazepines (BDZs) are commonly prescribed drugs for anxiety disorders and are considered first line of therapy to treat anxiety disorders. There are numerous adverse effects associated with BDZs (Committee, 2008)). Long or short term use of BDZs can cause severe adverse effects including anterograde amnesia, psychomotor impairment, memory disruption, dysarthria (a speech disorder), ataxia, impaired psychomotor function, paradoxical anxiety or aggression, risks of accidents and even mortality (Lader, 1999). Adverse effects of BDZs are one of the major reasons for patients to favor herbal alternatives over conventional medicines (Del Mundo et al., 2002). Thus, a strong need for safe anxiolytic alternatives over those currently used therapy is called for.

Traditionally, several herbal medicines have been used for the alleviation of anxiety and stress, though few of the herbs have been well characterized, and there is limited scientific evidence for their efficacy (Sarris et al., 2011). *Scutellaria lateriflora* (American skullcap) has long been an important herb in traditional medicine practices to treat anxiety and related disorders (King and Felter, 1909). It has been considered as one of the most valuable nervines (substance supporting the nervous system) by Americans and Europeans (Millspaugh, 1892). Clinical studies validated therapeutic benefits of *S. lateriflora* in anxiety disorders (Brock et al., 2013; Wolfson and Hoffmann, 2003). Furthermore, the demand for *S. lateriflora* in world markets is growing (Greenfield and Davis, 2004). A double blind, placebo-controlled clinical trial indicated that commercial preparations *of S. lateriflora* have therapeutic benefits in anxiety with no evidence of toxicity (Wolfson and Hoffmann, 2003). Another study determined that flavonoids and γ-aminobutyric acid are responsible for anxiolytic activity of *S. lateriflora* (Awad et al., 2003).

Several studies found that phytochemicals, scutellarein and ikonnikoside of *S. lateriflora* have benzodiazepine (BDZ) and serotonin-7 (5HT7) receptor binding affinity in

brain tissue (Gafner et al., 2003; Hui et al., 2000; Liao et al., 1998). The 5-HT7 receptor is thought to play a role in both the pathogenesis and prevention of various ailments such as, sleep disorders, depression, and migraine. AMPAR and NMDAR-subtype glutamate, and GABAR in neurons play important roles in synaptic plasticity. However no study to date has examined the effects of *S. lateriflora* on memory function. Therefore, the objectives of this study were to examine the effects of *S. lateriflora* on long term potentiation and, activities and expressions of NMDAR, AMPAR and GABAR in neurons.

6.2. Materials and Methods

Preparation of acute hippocampal slices and synaptosomes: Acute hippocampal slices were prepared according to previously described procedures (Parameshwaran et al., 2012; Parameshwaran et al., 2007b). Male Sprague-Dawley rats (1 month of age) were sacrificed in CO_2 chamber and brains removed. Brains were immediately submerged in ice-cold cutting buffer, and aerated with 95 % O_2 and 5 % CO_2. Cutting buffer contained 85 mM NaCl, 2.5 mM KCl, 4 mM $MgSO_4$, 0.5 mM $CaCl_2$, 1.25 mM NaH_2PO_4, 25 mM $NaHCO_3$, 25 mM glucose, 75 mM sucrose, 0.5 mM ascorbate and 2 mM kynurenic acid, with pH adjusted to 7.4. Hippocampal slices of (400 µm thickness) were prepared by using a tissue sectioning vibrotome on brains (Technical products international Inc., St. Louis, MO, USA). The anatomical accuracy of hippocampal slices was rectified using microscope. Hippocampal slices were incubated for two hours in artificial cerebrospinal fluid at 30 $^{\circ}$C aerated with 95 % O_2 and 5 % CO_2. Artificial cerebrospinal fluid contained 119 mM NaCl, 2.5 mM KCl, 1.3 mM $MgSO_4$, $CaCl_2$ 2.5 mM, 1 mM NaH_2PO_4, 26 mM $NaHCO_3$ and 11 mM dextrose. Synaptosomes (spherical synaptic terminal portion of nerve cell containing active receptors) were prepared according to previously described method by (Suppiramaniam et al., 2006). Some of the hippocampal sections were homogenized in ice-cold, modified Krebs-Henseleit buffer (mKRBS). The buffer contained 118.5 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.18

mM $MgSO_4$, 1.18 mM KH_2PO_4, 24.9 mM $NaHCO_3$, 10 mM dextrose, and a mixture of protease inhibitors containing 10 mg/ml of adenosine deaminase, 0.01 mg/ml leupeptin, 0.005 mg/ml pepstatin A, 0.10 mg/ml aprotinin and 5 mM benzamide, to avoid tissue proteolysis. The buffer was oxygenated with 95% O_2 and 5% CO_2 and the pH of mKRBS buffer was adjusted to 7.4. Hippocampal sections were homogenized in 500 µl ice-cold mKRBS buffer using homogenizer (Sigma-Aldrich, St Louis, MO). Homogenate was loaded into a 1 ml syringe, passed through a 100 µm pore size of nylon filter pre-wetted with mKRBS buffer, followed by filtration through 5 µm pore size of low protein binding filters, also pre-wetted with mKRBS. Filtered were centrifuged at 1000 xg for 15 min at 4°C and supernatant was discarded. The pellet, which contained synaptosomes, was re-suspended in 20 µl of mKRBS buffer and stored at -80° C until further use. All chemicals, except where noted, were purchased from Sigma-Aldrich (St Louis, MO)

Recording of field excitatory postsynaptic potentials from hippocampus slices: Transverse brain slice was held between two nylon nets and submerged in artificial cerebrospinal fluid and was continuously perfused the brain slice with 95 % O_2 and 5 % CO_2 at a flow rate of 3 ml/minutes. Electrical stimulation (0.33 Hz) was delivered for a brief period (<200 µs) to the Schaffer-Collateral Commissural Pathway of the Hippocampal CA1 region by placing a platinum electrode in the stratum radiatum. A glass electrode (1-4 MΩ) filled with artificial cerebrospinal fluid was used for recording field excitatory postsynaptic potentials (fEPSP) from the CA1 region and were recorded by with a Axoclamp (Axon Instruments, Foster city, CA). In long term potentiation (LTP) experiments, the slices of hippocampus were treated with alcoholic extract of *S. lateriflora* (SLE) at 5µg/ml concentration or vehicle control (PBS) just before electrophysiological recording. Alcoholic extract of *S. lateriflora* was prepared as described in chapters 3 and 4 of this dissertation (Lohani et, 2013). Stable baseline potential was recorded for at least 15 minutes at 50% of maximal response and then

theta burst (high frequency repetitive magnetic/electrical stimulation used to induce LTP) (10 bursts of stimuli, each burst of four pulses at 100 Hz) electric stimulation were delivered every 200 msec to afferent fibers of Schaffer-Collaterals, and field excitatory post synaptic potential (fEPSP) for each treatment group (n = 8) were measured in stratum radiatum using a recording electrode (Plastics One, Inc., Roanoke, VA, USA). Total peak amplitude and slope of fEPSP for control and SLE treated hippocampal slices were analyzed using Win-LTP software (version 2.1, Bristol, UK). A two-tailed unpaired t-test was used to determine differences in slope of fEPSP between control and SLE (n=8) treated hippocampal slices at significance at $p<0.05$ significance.

Reconstitution of synaptosomal receptors in phospholipid bilayers and measurement of single channel activities of excitatory NMDA, AMPA and inhibitory GABA receptors: Synaptosomes were reconstituted in a small "tip-dip" (referring to tip of glass pipette dip into artificial lipid bilayers, therefore tip of glass pipette containing lipid bilayers) artificial lipid bilayers (Suppiramaniam et al., 2006). Phospholipid (1, 2–diphytanoyl-sn-glycero-3-phosphocholine, final concentration 1 mg/ml) was dissolved in hexane to prepare synthetic phospholipids. Synthetic phospholipids were suspended in a buffer consisting of 125 mM NaCl, 5 mM KCl, 1.25 mM NaH_2PO_4, and 5 mM Tris HCl. Five microliters of phospholipid suspension was put into 300 µl of bath solutions containing artificial extracellular solution. Artificial extracellular solution contained 125 mM NaCl, 5 mM Tris HCl, 5 mM KCl, and1.25 mM NaH_2PO_4. The phospholipid bilayer was formed in the tip of a glass pipette (100 MΩ) and loaded with artificial intracellular buffer solution containing 110 mM KCl, 4 mM NaCl, 2 mM $NaHCO_3$, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 2 mM 3-N-Morpholino propanesulfonic acid (MOPS). Phospholipid bilayers were formed by successive transfer of two monolayers into the tip of the pipette. The pH of the solutions was maintained at 7.4. Three microliter suspensions of the synaptosomes were delivered into the artificial

extracellular solutions after membranes were stabilized. Affects of SLE on specific receptor activities was examined in the presence of all channel blockers except channel of interest. A hundred micromolar of (2R)-amino-5-phosphonovaleric acid, 1μM of 4-(4-aminophenyl)-1, 2-dihydro-1-methyl-2-propylcarbamoyl-6,7 methylenedioxyphthalazine, 1μM of (2S, 4R)-4-methylglutamic acid, 100 μM of picrotoxin, 2 mM of tetraethylammonium chloride, and 1 μM of channel blocker tetradotoxin which block NMDAR, AMPAR, kainite, γ aminobutyric acid, and glycine receptors, and potassium and sodium channels, respectively. Data exhibiting long stretches of single channel current transitions (consisting of negative or positive drift) without major base line drifts were chosen for quantitative analysis. All points-current amplitude histograms were constructed and fitted with the Gaussian method to identify and measure individual conductance levels. Currents of single channel AMPAR, NMDAR or GABAR were amplified in order to be able to measure each amplitude (Axopatch 200 B, Molecular Devices, Foster city, CA), filtered at 2 kHz, sampled at 20 kHz, digitized and recorded using a PCM interface (VR-10B Digital Data Recorder, Instrutech Corp., Elmont, NY). Data were analyzed offline using pCLAMP 9.0 software (Molecular Devices, Sunnyvale, CA). Single channel open probability was computed by combining area under current-amplitude histogram. Histograms of receptor dwell time measurement after log-transformation were constructed and fitted with variable metric fitting method to identify distinct open and close times.

Western blot : Undifferentiated hippocampal H19-7 cells were propagated up to 80-90 % confluency in 10 cm^2 culture plates. After harvested with 0.25% trypsin (w/v, in PBS), these undifferentiated cells were re-plated into poly-L-lysine coated 96-well plates at a density of 2 $\times 10^5$ cells/ml. Once H19-7 cells reached 80% confluency in wells, they were allowed to differentiate for 24 hours. These differentiated cells were incubated with a range of concentrations (0-10 μg/ml) of SLE for 24 hours to measure the effects of non-toxic

concentrations of SLE determined in previous experiment (refer to chapter 4) . At the end of the incubation period, the cells were harvested by centrifugation, washed with phosphate buffer saline (PBS) and lysed in a buffer solution containing 50 mM 2-amino-2-hydroxymethyl-1,3-propanediol chloride (Tris-HCl, pH 7.4), 150 mM sodium chloride, 1 % Nonidet P-40, 0.5 % sodium deoxycholate, 0.1 % sodium dodecyl sulfate, and a cocktail of protease inhibitors. Protein concentration of each sample was measured using the Thermo Scientific Pierce 660 nm Protein Assay reagent. Protein samples were aliquoted into 50 μg fractions and stored at -70 ºC. On the day of the experiment samples were boiled in sample loading buffer for 5 minutes, loaded onto a gradient of 4-12% NuPAGE Bis-Tris Gel (Life Technologies, Grand Island, NY) and electrophoretically transferred onto a polyvinylidene difluoride (PVDF) membrane via the wet transfer method. Non-specific binding sites on the membranes were blocked with 5% fat-free milk in Tris-buffered saline plus 0.1% Tween-20, and pH kept at 7.4 (TBST). The membranes were probed with anti-GABAR1, anti-GluR1, anti-NR2A and anti-NR2B antibodies (Cell Signaling Technology, Beverly, MA) along with anti β-Actin (Cell Signaling Technology, Beverly, MA), which was used as the loading control. Membranes were washed with TBST (3 times, each for 10 min) and incubated with specific Horseradish Peoxidase-conjugated secondary antibodies (Cell Signaling Technology, Beverly, MA) for 60 min at room temperature. Membranes were again washed three times for 10 min with TBST after incubation with each antibody. After washing, membranes were incubated with chemiluminescent reagent for 5 minutes in order to quantitate antibody. Membranes were wrapped in plastic wrap and placed in the x-ray film cassette with protein side next to the film. The chemiluminescence signals were measured as the band density obtained on the X-ray film through utilization of Quantity One software (Bio-Rad Laboratories, Hercules, California). The band densities for each sample was normalized to their β-actin signal and reported as a percentage control.

6.3. Results

Effects of SLE on long term potentiation (LTP) in hippocampal slices: Long-term potentiation is considered as the synaptic model of learning and memory in-vitro and is induced by theta-burst stimulation (TBS) at synapses in hippocampal slices after treatement with phosphate buffered saline or SLE. The TBS protocol electrical stimulation involves 5 trains (electric stimulation) of 10 bursts composed of 4 pulses at 100 Hz, with an inter-burst period of 200 ms. Theta-burst stimulation has been considered the most effective method to induce LTP as it instigates the normal physiological discharge characteristic of neurons (Albensi et al., 2007). Long term potentiation (LTP) was significantly decreased in eight of eight hippocampal slices that were treated with 5 µg/ml concentration of SLE (Figure 6.1, $p < 0.05$; n= 8), with an average of 133.7 % ± 6.748 %, compared with control slices (phosphate buffered saline vehicle) in which LTP was induced and stably maintained in eight of eight slices with an average of 153.6 % ± 0.2189 % in saline-control slices.

Effects of SLE on channel activities in synptosomes: Most of the excitatory (NMDAR, AMPAR) and inhibitory (GABAR) neurotransmission in the hippocampus is mediated by glutamatergic and γ-aminobutyric acid components, which play important roles in synaptic plasticity. In our first experiment we found that SLE decreased LTP which may be due to increased activities of GABAR channels as GABA amino acid is a major inhibitory amino acid of SLE. Alcoholic extract of *S. lateriflora* increased proportion of open stimulatory AMPARs channels but did not affect channel's conductance as shown in representative traces and current amplitude histograms (Figure 6.2 A and 6.2 B). Results showed that AMPAR channel open probability (P_o) was increased by SLE treatment ($P_o = 34.45$ % ± 0.67 %) compared to control (Figure 6.2 C, $P_o = 24.62$ % ± 0.83 %; n = 5, $p^{***} < 0.001$). Marquart least squares methods were used to calculate channel open and close dwell times by generating bimodal histograms. The open time of AMPARs channel was increased by SLE

treatment (τ_1: 0.774 ± 0.043 ms; τ_2: 8.668 ± 0.352 ms) compared to vehicle treated control (τ_1: 0.654 ± 0.031 ms; τ_2: 6.952 ± 0.6729 ms,) (Figure 6.2 D, n = 5). AMPARs dwell close time was unaffected by SLE treatment (τ_1: 0.853 ± 0.075 ms; τ_2: 18.64 ± 3.01 ms) compared to control (τ_1: 0.831 ± 0.063 ms; τ_2: 24.37 ± 4.24 ms) (Figure 6.2 E, n= 5). Conductance of AMPAR channels was not affected by SLE (28pS) as compared to control (29 pS). Proportion of open NMDARs channels was increased by SLE treatment but conductance of NMDARs channels was not affected by SLE as compared to vehicle control (Figure 6.3 A and 6.4 B). Open probability (P_o) of NMDAR channels were increased by SLE treatment (P_o = 34.38 % ± 1.58 %) compared to vehicle treated control (P_o = 24.71 % ± .8176 %) (Figure 6.3 C, $p** < 0.01$, n = 5). The open time of NMDAR channels were increased by SLE treatment (τ_1: 0.6994 ± 0.0302 ms) compared to control (τ_1 : 0.5496 ± 0.0470 ms) (Figure 6.3D, $*p<0.05$, n = 5). Similar to open time, close time of NMDAR channels was also modulated by SLE treatment (τ_1 : 0.94 ± 0.04 ms; τ_2 : 21.70 ± 1.14 ms) compared to control (τ_1: 1.38 ± 0.1302 ms; τ_2: 14.97 ± 1.72 ms) (Figure 6.3 E, $**p<0.01$, n = 5). The conductance of NMDA channels was not affected by SLE as compared to control (40 pS). Representative traces and current amplitude histograms of GABAR channels showed SLE increased numbers of open GABARs channels of their conductance (Figure 6.4 A and 6.4 B)Open probability (P_o) of GABA channels was increased by SLE treatment (P_o = 34.45 % ± 0.679 %) compared to control (P_o = 14.62 % ± 0.837 %) (Figure 6.4 C, n = 5, $p**** < 0.0001$). The conductance of GABA channels was significantly increased by SLE treatment (60.00 pS) as compared to control (30.00 pS). The open dwell time of GABAR channels was significantly increased by SLE treatment (τ_1: 0.6295 ± 0.0144 ms, τ_2 : 6.25 ± 0.30 ms) compared to control (τ_1 : 0.3837 ± 0.0252 ms ; τ_2 : 3.76 ± 0.19 ms) (Figure 6.4 D, $***p < 0.001$; n = 5). In contrast to open time, close time of GABA was decreased by SLE treatment

(τ_1 : 0.7886 ± 0.0481 ms; τ_2 : 16.21 ± 0.4468 ms) compared to control (τ_1: 1.0650 ± 0.0416 ms; τ_2 : 26.89 ± 1.224 ms) (Figure 6.4 E, n = 5)

Effects of SLE on glutamate and GABA receptors 'expression: Alcoholic extract of *S. lateriflora* did not affect N-methyl-D-aspartate receptor (NMDA) receptor subunits NR2A and NR2B proteins, α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor subunit GluR1 and gamma-aminobutyric acid subunit GABA R1 protein expression in differentiated H19-7 cells compared to vehicle treated control (Figure 6.5).

6.4. Figures and Figure legends
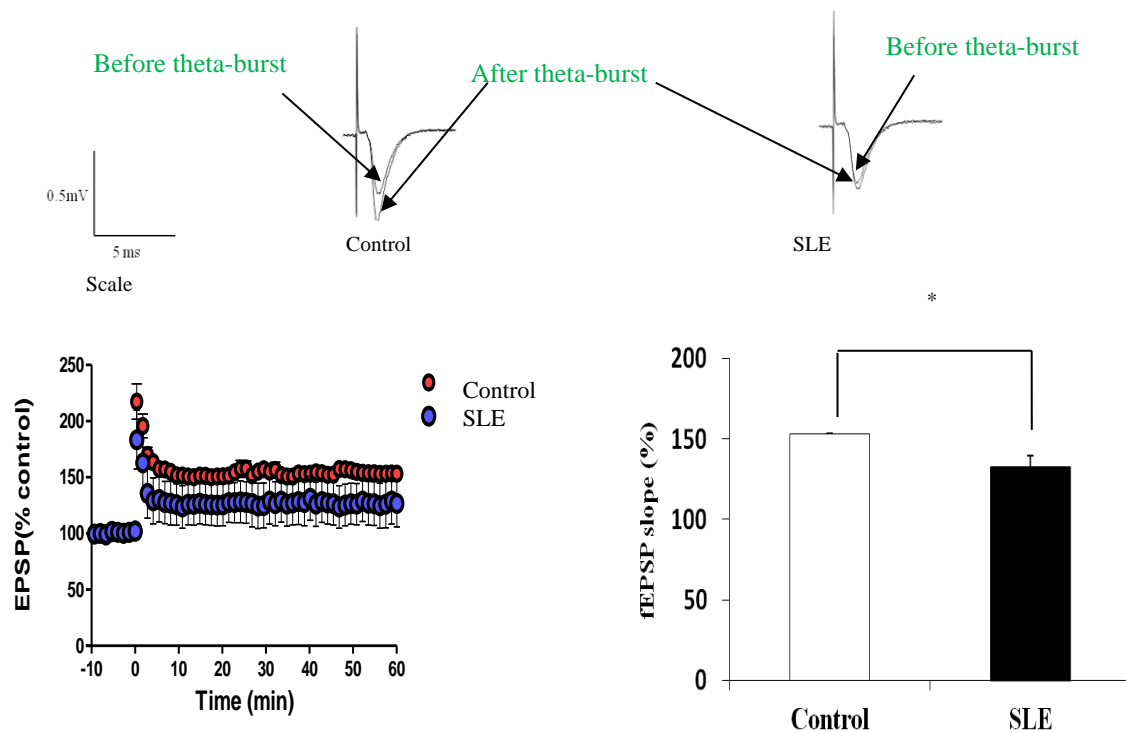
Figure 6.1. Effect of SLE exposure on hippocampal long term potentiation (LTP). LTP was induced by electrical theta burst stimulation (TBS) and measured at 55-60 min after TBS. LTP in SLE treated hippocampal slices were significantly reduced compared to control. Normalized fEPSPs (field excitatory post synaptic potential) slopes of 60 min post-TBS averaged 153.6% ± 0.2189 % for control and 133.8% ± 6.748% for SLE treated

Figure 6.2

A

Control

Conductance (pS) = 29

Po = 24.62 % ± 0.83 %

3 pA

200 ms

SLE (5 µg/ml)

Conductance (pS) = 28

Po = 34.45 % ± 0.67 %

3 pA

200 ms

B

Control

Open channels   Close channels

SLE (5 µg/ml)

Open channels   Close channels

C

***

D

Open time AMPAR

*

E

Close time AMPAR
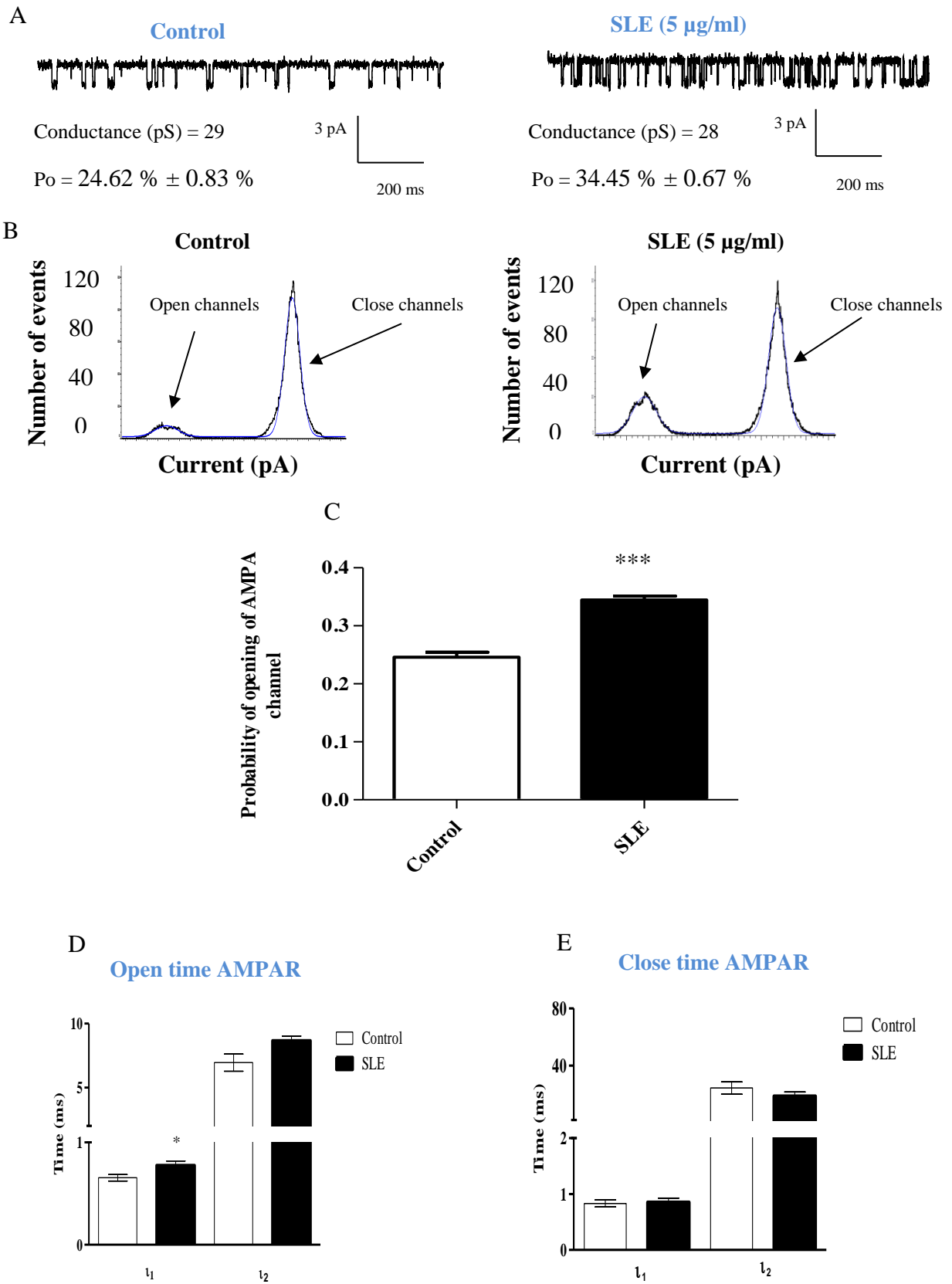
Figure 6.2 SLE exposure is associated with alterations in the single channel properties of hippocampal synaptic excitatory AMPARs**.** Representative traces **(A)** and current amplitude histograms **(B)** are shown for control (left) synaptosomes and SLE (right) treated synaptosomes. The channel conductance of excitatory AMPARs currents were not affected by SLE treatment (28 pS) compared to control (29 pS). **(C)** Bar plots showing significantly increased channel open probability (P_o) in SLE treated synaptosomes compared to the control. **(D)** Bar plots illustrating increased open times (only $\tau 1$ but not $\tau 2$) of AMPA channels by SLE treatment. **(E)** Bar plots showing close times ($\tau 1$ and $\tau 2$) of AMPA channels were unaffected by SLE treatment (n = 5, ***$p < 0.001$ versus control by two tailed student's t test.

Figure 6.3

A

Control

Conductance (pS) = 40

Po = 24.71 % ± 0.8176 %

3 pA

200 ms

SLE (5μg/ml)

Conductance (pS) = 39

Po = 34.38 % ± 1.58 %

3 pA

200 ms

B

Control
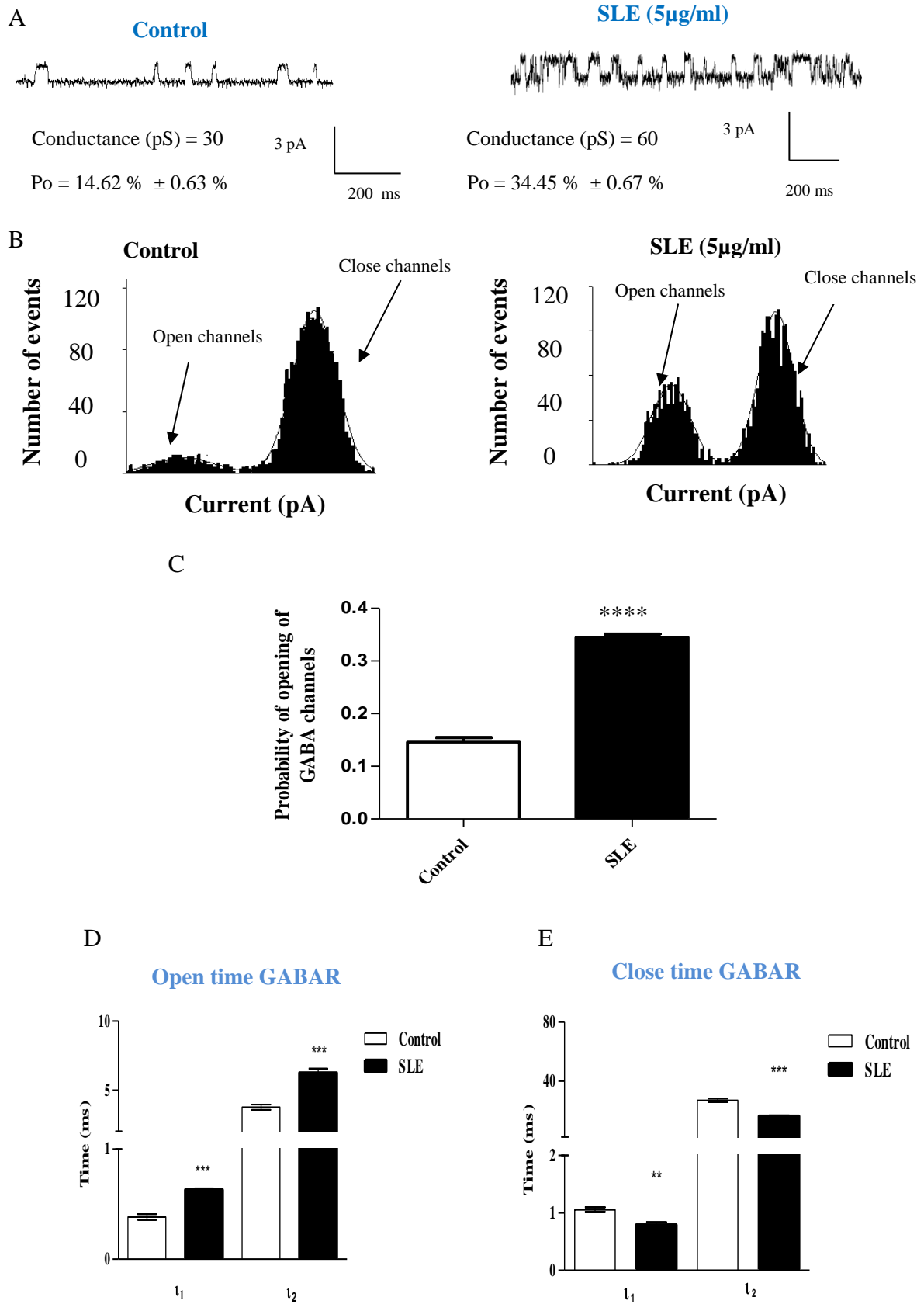

SLE (5μg/ml)

C

D

Open time NMDAR

E

Close time NMDAR
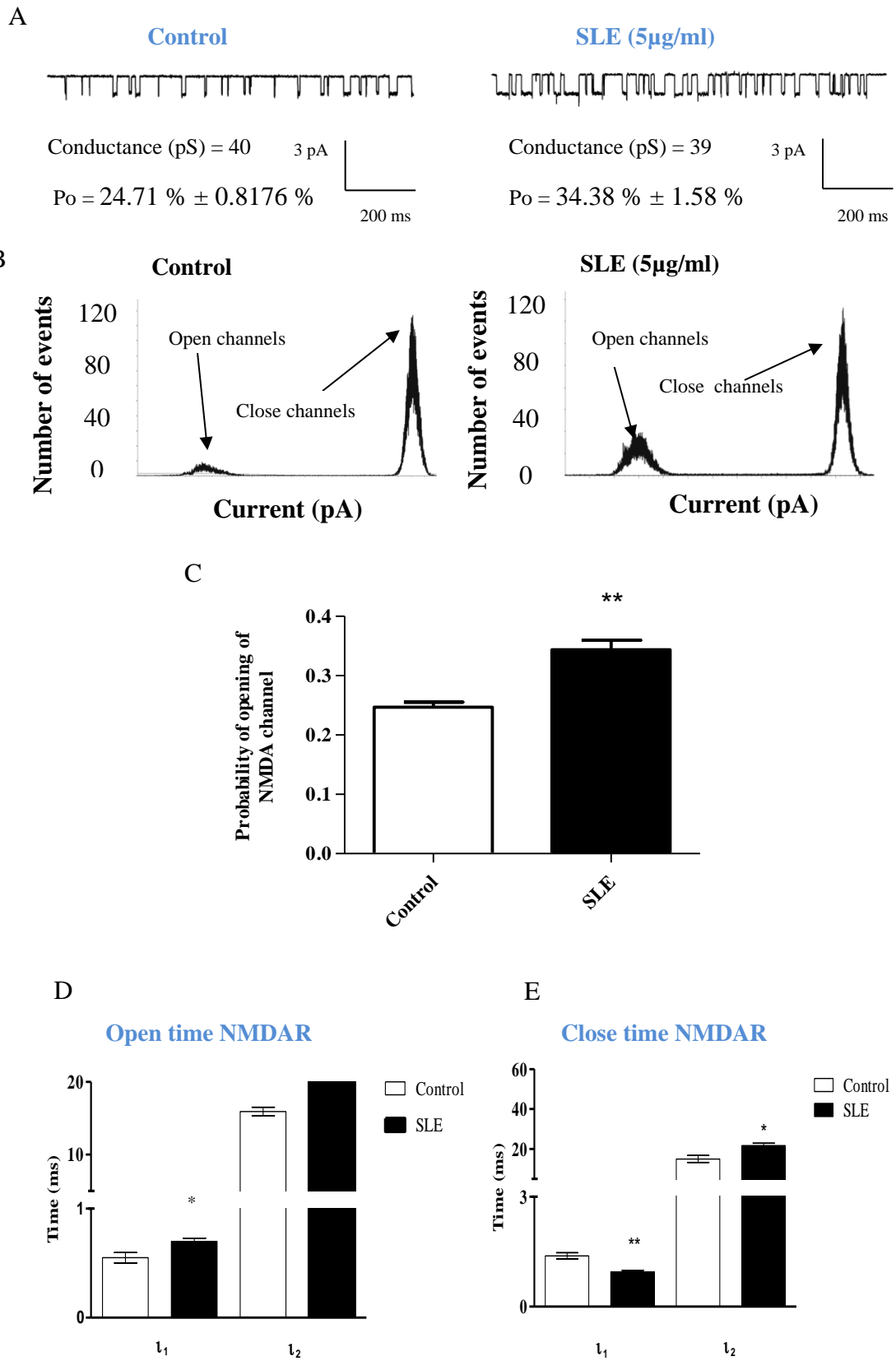
Figure 6.3 SLE exposure is associated with alterations in the single channel properties of hippocampal synaptic NMDARs. Representative traces **(A)** and current amplitude histograms **(B)** are shown for control (left) synaptosomes and SLE (right) treated synaptosomes. The channel conductance of NMDARs currents were not affected by SLE treatment (39 pS) compared to control (40 pS) **(C)** Bar plots showing significantly increased channel open probability (P_o) in SLE treated synptosomes compared to the controls. Bar plots illustrating open times ($\tau 1$ but not $\tau 2$) **(D)** were increased by SLE treatment and modulated close times (decreased $\tau 1$ but increased $\tau 2$) **(E)** of NMDA channels by SLE treatment (n = 5, *p<0.05 or **p<0.01 versus control by two tailed student's t test)

Figure 6.4

A

Control   **SLE (5µg/ml)**

Conductance (pS) = 30   Conductance (pS) = 60

Po = 14.62 % ± 0.63 %   Po = 34.45 % ± 0.67 %

3 pA

200 ms

B

C

D   E

Open time GABAR   **Close time GABAR**

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Figure 6.4 SLE exposure is associated with increased single channel activities of hippocampal synaptic GABARs. Representative traces **(A)** and current amplitude histograms **(B)** are shown for control (left) synaptosomes and SLE (right) treated synaptosomes. The channel conductance of GABARs currents were increased by SLE treatment (60 pS) compared to control (30 pS) **(C)** Bar plots showing significantly increased channel open probability (P_o) in SLE treated synptosomes compared to the controls. Bar plots illustrating open times **(D)** were increased but decreased close times ($\tau 1$ and $\tau 2$) **(E)** of GABA channels by SLE treatment (n = 5, **p<0.01, ***p<0.001 or ****p<0.0001 versus control by two tailed student's t test)
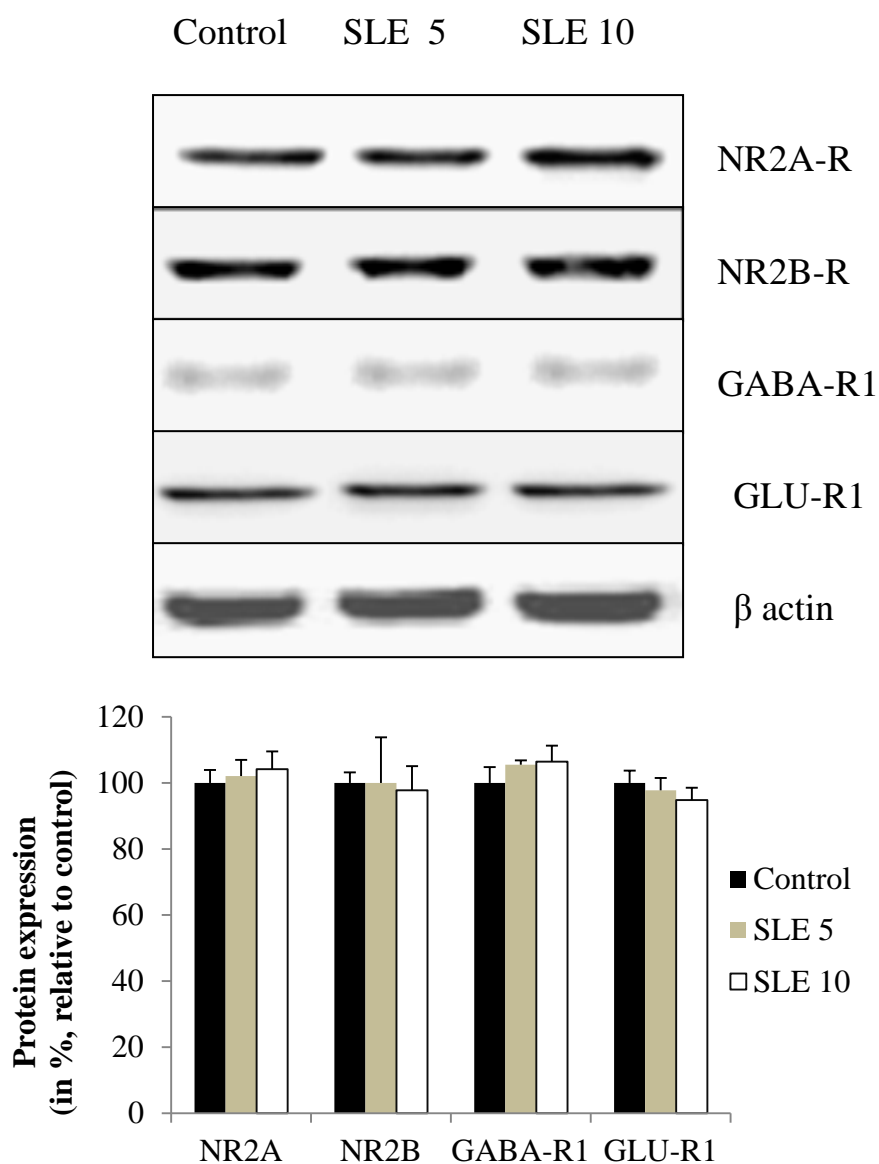
Figure 6.5 Effects of SLE on protein expression of NMDA-R (NR2A and NR2B), AMPA-R (GLU-R1) and GABA receptors in differentiated hippocampal (H19-7) cells. Undifferentiated H19-7 cells were grown in poly-L-lysine coated 10 cm^2 plates up to 70-80% confluency and then allowed them to differentiate by incubating in differentiating media for 24 hours. Differentiated cells were then incubated with various concentration of SLE for next 24 hours. SLE 5 and SLE 10 indicate 5 and 10 μg/ml concentration of alcoholic extract of *Sctellaria lateriflora*. Protein expressions are expressed relative to control (phosphate buffered saline) control. Each value represents the mean ± SEM, (n=3) obtained from 3 different independent experiments.

6.5. Discussion

Scutellaria lateriflora, a traditional herbal remedy for stress and anxiety, was tested on human volunteers for its effects on mood. A 350 mg dose of ground, dried aerial portion of *S. lateriflora* given three times daily for over two weeks mildly suppressed anxiety in patients (Brock et al., 2013). *Scutellaria lateriflora* did not exhibit any adverse reactions, tolerance, dependability or rebound excitability in mildly anxious human subjects (Brock et al., 2013). However no study to date analyzed the effects of *Scutellaria lateriflora* on memory functions, as anterograde amnesia is the most common adverse affects of long or short term use of benzodiazepine, which is considered the first line of therapy for these patients (Lader, 1999) .Therefore, the effects of *S. lateriflora* on memory function and potential mechanisms by which *S. lateriflora* modulates synaptic transmission were analyzed.

Axons and dendrites are nerve cell projections through which action potential (signal) transmits between two neurons. The terminal area of one neuron makes contact with the terminal portion of a dendrite of another neuron, and these contact areas are known as synapses. Synapses are sites where one neuron communicates with another neuron through chemical signal transmission. A long-lasting enhancement of signal transmission between two neurons is known as long term potentiation (LTP) and it results from continuously stimulating presynaptic neurons. LTP is widely considered as one of the major cellular mechanisms that underlie learning and memory (Bliss and Collingridge, 1993; Cooke and Bliss, 2006) . We found that of LTP was partially decreased by SLE treatment compared to vehicle control in rat hippocampi. Suppressed hippocampal LTP may lead to the cognitive decline (Rowan et al., 2003) . A previous research study found mild reduction in cognition by *S. lateriflora* (Wolfson and Hoffmann, 2003). However, the results of a recent study on human subjects contradicted these findings and suggested that administration of *S. lateriflora* did not impair cognitive function (Brock et al., 2013). N-methyl-D-aspartate

(NMDARs) and α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors (AMPARs) are two major types of ionotropic glutamate receptors. Activation of these receptors results in basal excitatory synaptic transmission and many forms of synaptic plasticity such as LTP mechanisms (Citri and Malenka, 2008; Lynch, 2004; Malenka, 1994). In fact, single channel activities of glutamate receptors (NMDARs and AMPRs) are essential for synaptic plasticity in terms of LTP. (Ambros-Ingerson and Lynch, 1993; Benke et al., 1998; Parameshwaran et al., 2007a; Wijayawardhane et al., 2008). In contrast to glutamate receptors, activation of inhibitory GABA receptors led to impairment of LTP (Bliss and Collingridge, 1993; Kleschevnikov et al., 2004). We found that alcoholic extract of *S. lateriflora* increased excitatory postsynaptic AMPA and NMDA receptor probabilities of being open, and their receptors open time compared to vehicle treated synaptosomes. However, SLE did not change conductance (a measure of easiness with which ions pass through a receptor) for AMPA and NMDA channels compared to control. Presence of glutamate in SLE was probably responsible for activation AMPA and NMDA receptors as it has been reported that glutamate constitutes approximately 0.34% of dry weight of alcoholic extract in *S. lateriflora* (Bergeron et al., 2005). In contrast to glutamate receptors, SLE significantly increased conductance of GABA receptors compared to control. Additionally, SLE increased open channel probability and open time, but decreased close time of GABARs compared to control. SLE increased open channel probability, conductance of GABARs to a greater extent than to glutamate receptors. Therefore, increased inhibitory GABA receptors conductance decreased strength of fEPSP by SLE. Activation of GABA receptor by SLE is attributed to the presence of polyphenolic flavonoids and γ-Aminobutyric acid (GABA) (Awad et al., 2003). Gamma-aminobutyric acid (GABA) is major inhibitory neurotransmitter, that constitutes approximately 0.55 % of dry weight of alcoholic extract in *S. lateriflora* (Bergeron et al., 2005). It has been reported that baicalin and baicalein, major flavonoids

present in *S. lateriflora*, are known to bind to the benzodiazepine site of the GABAA receptor (Awad et al., 2003).

Increased protein expression of excitatory and inhibitory receptors in hippocampus cells could be modulated by SLE treatment therefore, protein expression of glutamate and GABA receptors was determined in SLE treated hippocampal cells. It has been found that SLE did not increase expression of NMDA receptors (subunits NR2A and NR2B), AMPA receptor (subunit GLUR1) and GABA receptor (subunit GABAR1) expression in differentiated hippocampal cells compared to vehicle control.

In conclusion, the results of our in-vitro experiments suggest that *S. lateriflora* decreased synaptic plasticity by enhancing GABA receptor activities without increasing their protein expression. Enhancing GABA receptors activity of SLE is, at least a part, attributed to its high GABA and flavonoids content as both binds to GABA receptors. However, it could be that results of our in-vitro experiments will not be applicable to animal or human subjects as inhibitory neurotransmitter GABA cannot pass through blood brain barrier and not accessible to neurons. Therefore, it is conceivable that SLE does not decreased synaptic plasticity in animals or human subjects. Furthermore, in contrast to anxiolytic BDZs that block LTP formation as reported by previously published research articles (Del et al., 1992), LTP was induced and maintained in SLE treated hippocampus brain slice suggesting its lesser adverse effects on memory functions. Therefore, *S. lateriflora* is a better anxiolytic therapeutic compare to commonly use BDZs, in terms of severity of adverse effects. However, further in vivo studies are required to explore affects of *S. lateriflora* on synaptic plasticity.

6.6. References

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7. Summary

American skullcap (*Scutellaria lateriflora*) is a native plant of North America and contains chemically similar active flavonoids that are found in Chinese skullcap (*Scutellaria baicalensis*). However, in spite of the facts that aerial parts of *Scutellaria lateriflora* contains higher concentrations of flavonoids than Chinese skullcap, very limited research studies have been done to examine pharmacological activities of American skullcap to date. In our experiments we determined anti-oxidative, anti-inflammatory, and immunomodulatory, and gamma amino butyric acid (GABA) receptor activities of alcoholic extract of *Scutellaria lateriflora* (SLE). The concentrations required to reduce 50% (IC50 value) thiobarbituric acid reactive substances (a product of lipid peroxidation) formation compared with the vehicle control was determined to be 350 µg/mL for SLE compared with the positive control alpha-tocopherol (IC50 = 325 µg/mL). Both the SLE and the positive control, ascorbic acid, at 125 µg/mL scavenged more than 80% reactive oxygen species (ROS) generation. Furthermore, the SLE protected hydrogen peroxide-UV induced cleavage of supercoiled plasmid DNA. The neuroprotective properties of SLE were evaluated in hydrogen peroxide-induced oxidative stress in differentiated hippocampal H19-7 cells. SLE suppressed caspase-3 pro-apoptotic expression and scavenged the ROS of hydrogen peroxide-induced oxidative stress. Decreased ROS and caspase-3 activity by SLE extract strongly associated with the increased cell viability. Additionally, SLE increased anti-oxidant protein brain derived nerve factor (BDNF, involved in neurogenesis) expression in H19-7 cells. The SLE inhibited inflammatory cyclooxygenase-1(COX-1) and cyclooxygenase-2 (COX-2) enzyme activities. The IC50 values for COX-1 and COX-2 were calculated to be 50 µg/mL and 125 µg/mL, respectively. We further determined effects of *Scutellaria lateriflora* on memory functions as long term use of currently used anxiolytic drugs have adverse effects on memory. Long-term potentiation is considered as the synaptic model of learning and memory in-vitro. Strength of

excitatory post synaptic potential in long term potentiation (LTP) was significantly decreased by SLE in hippocampal slices via enhancing inhibitory GABA receptors activity compared with control slices (phosphate buffered saline vehicle).

These findings indicate that *S. lateriflora* exhibit neuroprotection by its antioxidant and anti-apoptotic effects on neuronal cells. Additionally, our results indicate anti-inflammatory activities of SLE and therefore *S. lateriflora* is a potential treatment for diseases which have various forms of oxidative stress and inflammation involved in their pathogenesis. However, the results of our in-vitro experiments suggest that *S. lateriflora* decreased synaptic plasticity by enhancing GABA receptor activities. Increased GABA activities by SLE might, at least a part, be attributed to its GABA and flavonoids content since both bind to GABA receptors. Further experiments are needed to determine if effects on GABA receptors will be applicable to animal or human subjects because inhibitory neurotransmitter GABA cannot pass through the blood brain barrier and is not accessible to neurons in the CNS. Furthermore, stabilities and bioavailability of small molecular weight phytochemicals present in *S. lateriflora* in body is not yet known. Interactions of bioactive compounds with dietary constituents could also influence bioactive responses. Therefore, further in vivo studies are required to explore biological activities of *S. lateriflora*.