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

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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.

Keywords: *Scutellaria lateriflora*, Neuroprotection, Flavonoids, Antioxidants, Oxidative stress.

Oxidative stress in the brain is a potential pathogenesis for behavioral deficits and neurological disorders [1]. 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 catalase or superoxide dismutase. 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 [2, 3]. 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 [4] 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 [5]. 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 [6]. Gamma-aminobutyric acid constitutes approximately 0.55% of the dry weight of alcoholic extracts of *S. lateriflora* leaves and stem [7], which is considered to be responsible for its anxiolytic activity [8, 9]. 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 [7]. 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 [10]. 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. 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).

Table 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 ± 0.14[a]	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).

Direct radical scavenging activities of *S. lateriflora* were determined using the 2,2-diphenyl-1-picryhydrazyl (DPPH) radicals scavenging assay. Both ethanolic and aqueous extracts quenched stable 2,2-diphenyl-1-picryhydrazyl (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).

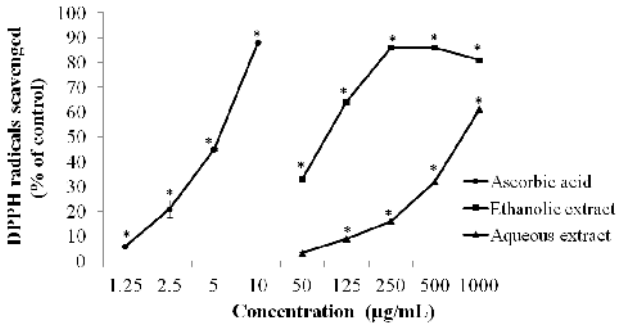

Figure 1: 2,2-diphenyl-1-picryhydrazyl (DPPH) radical scavenging effects of ethanolic and aqueous extracts of *S. lateriflora*. Results are expressed as percentage decrease of absorbance at 517 nm with respect to control (vehicle treated group). Ascorbic acid was used as a positive control. Each value represents the mean ± SEM, (n=6). * p <0.05 indicates significantly different from the control (vehicle treated) group.

Proxidant *t*-butyl hydroperoxide (1 mM) 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)) 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).

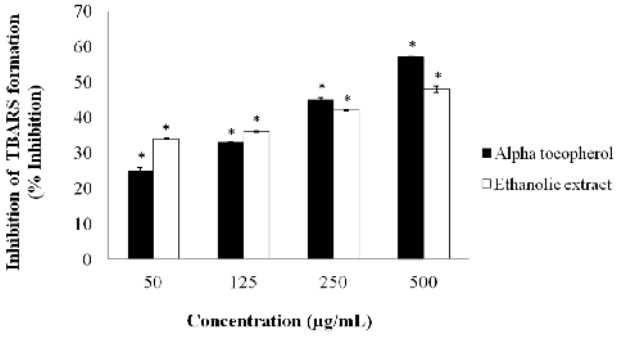

Figure 2: Ethanolic extract of *Scutellaria lateriflora* linearly inhibited hydrogen peroxide induced lipid peroxides level generation in mouse brain homogenates relative to vehicle control. α-Tocopherol was used as a positive control for lipid peroxidation generation assays. * p <0.05 indicates significantly different from the control (vehicle treated) group (n=6).

The lowest dose of the ethanolic extract of *S. lateriflora* and ascorbic acid scavenged more than 80% reactive oxygen species generation in the brain tissue homogenate. Lastly, we determined the effect of the flavonoid rich ethanolic extract of *S. lateriflora* on reactive oxygen species 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).

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

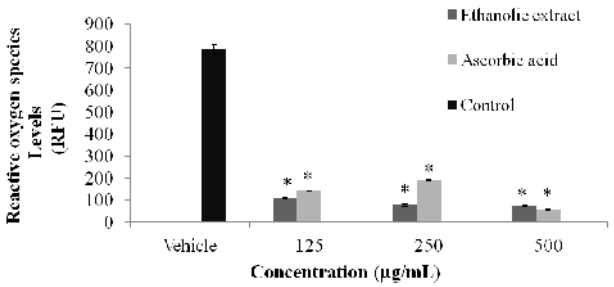

Figure 3: Ethanolic extract of *Scutellaria lateriflora* linearly inhibited hydrogen peroxide induced generation of reactive oxygen species in mouse brain homogenates relative to vehicle control. Ascorbic acid was used as a positive control for reactive oxygen species generation assays. * p <0.05 indicates significantly different from the control (vehicle treated) group (n=6).

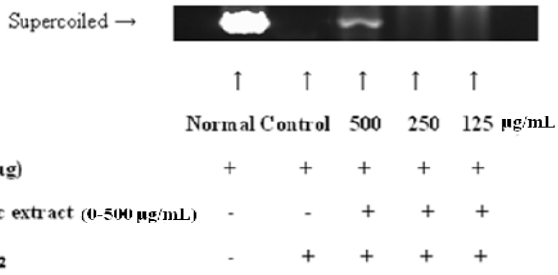

Figure 4: Ethanolic extract of *Scutellaria lateriflora* (0-500 µg/mL) protected plasmid DNA (Pbr322) against hydrogen peroxide and UV induced cleavage. Experiment was repeated three times. + or – indicates presence or absence of chemicals/compounds, respectively.

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 [4]. *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 [11]. Hence in this study, we investigated the neuroprotective mechanisms of *S. lateriflora.*

Imbalanced oxygen metabolism and excessive production of oxygen-derived reactive oxygen species (ROS) in neurons can lead to neurotoxicity [3,12]. 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 [13]. 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 [2, 14]. 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 [15].

Increased oxygen-derived reactive oxygen species generation due to cellular metabolism in neurons leads to toxicity in various neurological disorders [1,3,12]. Anxiety is one of the most prevalent neuropsychological disorders worldwide. Approximately, 18 percent of the adult population is affected by anxiety in the USA [16]. 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 [3]. There are several human and animal studies that have implicated the role of oxidative stress in anxiety [17,18]. Decreased (e.g., ascorbic acid) and / or increased antioxidant catalase activity and superoxide dismutase levels were observed in the cortex, hippocampus and blood of an animal model of anxiety [3,17-19]. Furthermore, vitamin E (tocopheral, lipid soluble antioxidant) deficiency can increase neuropsycological disorders in humans and rodents [3,20,21]. In general, patients with anxiety disorders have increased generation of reactive oxygen species, higher lipid peroxidation and DNA oxidation in the brain compared with healthy controls [19,22-24]. Effective use of dietary antioxidants has been reported to exhibit a cognitive enhancing effect, psychostimulant activity, and antidepressant properties [3,20]. Therefore, antioxidant rich dietary herbal medicine presents a potential effective and novel approach to the treatment of a range of neurological disorders [25]. 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.

Experimental

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 (Shorter, Alabama). The seeds were sown in the spring of 2007 and 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 dried in a forced-air dryer (Model AA-5460A) at 40^0C for 3 days after putting them in open paper bags. Finally, the dried aerial parts of plants were ground to a powder using a laboratory mill and stored at room temperature in large cloth bags that allowed movement of air until use. All other 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 [7]. Ground powder was either mixed in 70% aqueous ethanol or distilled water for 24 h at room temperature on a platform shaker. 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 -4^0C 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 [25]. Total phenol content was measured using the Folin-Ciocalteu method [26]. The total flavonoid content was determined using the previously described method [27].

Effect of* S. lateriflora *on DPPH radical scavenging activity: The 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay was performed as described previously [14] . *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 by 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: $I\%=(A_{blank}-A_{sample})/A_{blank}\times 100$ 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 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 [28]. 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**)** TCA and 400 µL of 0.5% (w/v, in PBS) thiobarbituric acid (TBA) were added to each tube, and incubated at 80^oC 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 [28].

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 [6]. After irradiation, 10 µL of sample was mixed with 2 µL of loading buffer and separated on agarose gels (1.5% w/v in TAE 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 $P<0.05$.

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