The butanol fraction of *Eclipta prostrata* (Linn) increases the formation of brain acetylcholine and decreases oxidative stress in the brain and serum of cesarean-derived rats

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**Abstract**

*Eclipta prostrata* has been used as a traditional medicinal plant to prevent dementia and to enhance memory in Asia. Its potential as a nootropic and as an antioxidant have been reported in mice. We hypothesized that *Eclipta* may affect the formation of neurotransmitters and the inhibition of oxidative stress. Charles River cesarean-derived rats (male, 180 ± 10 g) were fed experimental diets supplemented with 0 mg (control), 25 mg (E25), 50 mg (E50), or 100 mg (E100) of a freeze-dried butanol fraction of *E. prostrata* per kilogram of diet for 6 weeks. The acetylcholine level was significantly increased by 9.6% and 12.1% in the brains of E50 and E100 groups, respectively, compared with the control group that was fed standard diet alone. The acetylcholine esterase activity was significantly increased by 13.1% and 19.7% in the brains of E50 and E100 groups, respectively, compared with the control group. Monoamine oxidase-B activity was significantly decreased by 10.5% in the brains of the E100 group, and the superoxide radical level was significantly reduced by 9.4% in the serum of the E100 group compared with the control group. Superoxide dismutase activity was significantly increased by 13.1% and 19.7% in the brains of E50 and E100 groups, respectively, compared with the control group. These results clearly demonstrate the effects of *E. prostrata* on the formation of acetylcholine in the brain and the inhibition of oxidative stress in the brain and serum of rats. These findings may have implications for preventing dementia and enhancing memory function in humans.

**Keywords:** *Eclipta prostrata*; Rat; Neurotransmitter; Antioxidant; Antidementia

**Abbreviations:** ACh, acetylcholine; AChE, acetylcholine esterase; AD, Alzheimer disease; CD, cesarean derived; MAO, monoamine oxidase; MAO-B, monoamine oxidase-B; SOD, superoxide dismutase.

1. **Introduction**

Aging is a natural process that occurs in all living organisms and a multifarious event resulting from the collective effects of genetic variation, environmental risk factors, and nutritional factors. With age, bodies experience a
progressive deterioration of physical functions and loss of homeostasis, and become increasingly susceptible to disease [1]. The decrease in neurotransmitter formation and increase in oxidative stress that comes with aging affects brain functions such as memory, especially recognition, and causes dementia [2]. Acetylcholine (ACh) is the first substance proven to be a neurotransmitter [3] and has an important role in the enhancement of sensory perception when we wake up and in sustaining attention [5]. Acetylcholine esterase (AChE) is an enzyme that degrades the neurotransmitter ACh, producing choline and an acetate group [6]. An overall decrease of AChE in the brains of the people with Alzheimer disease(AD) has been observed [7-11]. The increase in free radicals that occurs with age would cause a progressive accumulation of cellular damage (DNA, protein, and lipid) [12]. Brains that consumed high levels of oxygen and so produced high levels of free radicals [13] were susceptible to oxidative stress and easily became aged. Therefore, aging affects brain functions such as memory including recognition and causes types of dementia including AD [14]. Elderly individuals with this disease are unable to take care of themselves and must rely on other family members or caregivers, thus resulting in a financial and psychological burden on society [15]. Currently, the US Food and Drug Administration approved drugs for AD treatments mainly provide symptomatic improvement by replacing neurotransmitters. However, they have little or no impact on the disease progress. Thus, natural antidementia material that can alter the disease progress has been of great interest to people [2].

Eclipta prostrata (Linn), a member of the Asteraceae plant family and commonly known as False Daisy, has been used as a traditional medicine to treat atherosclerosis, dementia, memory impairment, and hepatic disorders in Asia [16-19]. The potential neuropharmacological activity of the plant Eclipta as a nootropic has been reported. In the elevated plus maze, the extract significantly increased the exploration of the open arm. In the open field test, the extract significantly increased rearing, assisted rearing, and the number of squares traversed, all of which are demonstrations of exploratory behavior [19]. In our previous report, it was shown that the butanol fraction of Eclipta effectively reduces serum lipid levels and improves antioxidant activities in cesarean-derived (CD) rats [20,21]. Saponin, known as a major medicinal component of the butanol fraction of Eclipta, demonstrated a beneficial effect on memory impairment in mice and had a high antioxidant effect in vitro systems [22-24]. We hypothesized that Eclipta may have affected the formation of ACh and the inhibition of oxidative stress.

To determine the effects of Eclipta on these factors, including the formation of ACh and the inhibition of oxidative stress, we measured ACh content, AChE and monoamine oxidase-B (MAO-B) activities in the brain, levels of superoxide radicals, and superoxide dismutase (SOD) activity in serum after administration of Eclipta extract to CD rats.

2. Methods and materials

2.1. Animals and feeding studies

Forty Charles River Sprague-Dawley CD rats (Specific Pathogen-Free/viral antibody-free Crj/Bgi male, 180 ± 10 g) purchased from the Laboratory Animal Center (Biogenomics, Seongnam, Korea) were housed in stainless-steel wire cages and maintained on a 12-hour light/dark cycle in a temperature-controlled environment (22°C) with access to standard rat diet (Harlan Teklad, Madison, Wis) and water ad libitum for 2 weeks. Twenty-eight rats, which were individually housed in stainless-steel wire cages in the same environment, were randomly divided into 4 groups (7 rats per group) and fed 4 different experimental diets that consisted of a standard rodent diet [25] supplemented with high fat and high cholesterol [26,27] for 6 weeks. Experimental diets were prepared by mixing 25 mg (E25), 50 mg (E50), or 100 mg (E100) of freeze-dried E prostrata extract per kilogram of diet, which effectively reduced serum lipid levels and improved antioxidant activity in CD rats [20,21]. Controls were fed basal diet without E prostrata extract. All diets contained 18% protein, 58.3% carbohydrate, 15% fat, and 0.5% cholesterol (Table 1). The butanol fraction of E prostrata was prepared from crude methanol and successively extracted with chloroform, ethanol, ethyl acetate, and butanol. The final butanol fraction was concentrated by evaporation and freeze drying and stored at −20°C until use [21]. The experimental protocol was approved by the Institutional Animal Care and Use Committee of Pukyong National University.

2.2. Analytical procedures

After overnight fasting, euthanasia was carried out by cervical dislocation, and brain and blood samples were

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Ingredient composition of the control and experimental diets (g/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Composition</td>
<td>Control</td>
</tr>
<tr>
<td>Eclipta powdera</td>
<td>–</td>
</tr>
<tr>
<td>Cornstarch</td>
<td>45.0</td>
</tr>
<tr>
<td>Sucrose</td>
<td>13.3</td>
</tr>
<tr>
<td>Casein</td>
<td>18.0</td>
</tr>
<tr>
<td>Lard</td>
<td>10.0</td>
</tr>
<tr>
<td>Corn oil</td>
<td>5.0</td>
</tr>
<tr>
<td>Cellulose</td>
<td>3.0</td>
</tr>
<tr>
<td>AIN-76 mineral mixb</td>
<td>1.0</td>
</tr>
<tr>
<td>AIN-76 vitamin mixb</td>
<td>3.5</td>
</tr>
<tr>
<td>DL-Methion</td>
<td>0.3</td>
</tr>
<tr>
<td>Choline chloride</td>
<td>0.2</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>0.5</td>
</tr>
<tr>
<td>Sodium cholate</td>
<td>0.2</td>
</tr>
</tbody>
</table>

a Experimental diets contained 0 mg (control), 25 mg (E25), 50 mg (E50), and 100 mg (E100) of freeze-dried E prostrata butanol extract per kilogram of diet.

b AIN-76 mineral and vitamin mixtures were obtained from ICN (Solon, Ohio).
collected from the rats fed experimental diets for 6 weeks [20,21,26,27]. Brain samples were prepared using the method previously described [28], and serum was prepared by centrifugation at 3000 rpm for 10 minutes. In brief, brains (7 rats per group) were suspended in 0.1 mol/L TRIS buffer (Sigma, St. Louis, Mo) and homogenized for 1 minute using an Ultra Turrax (IKA Works, Inc, Wilmington, NC) [28]. Extracts were then centrifuged at 10,000 × g for 10 minutes, and the supernatants were used to determine ACh content, and AChE and MAO-B activity.

Brain ACh content, and AChE and MAO-B activity were analyzed using the methods previously described [29-31]. Acetylcholine content was measured by a modification of the procedure of Hestrin [29], which is based on the colorimetric determination of the red purple color produced by the binding between hydroxyamic acid and ferric ion. Fifty microliters of brain suspension and 50 μL of hydroxylamine (1%, Sigma) were mixed, the pH was adjusted to 1.2 using HCl (Sigma), and 500 μL of FeCl₃ (10% in 0.1N HCl) was added. ACh chloride (Sigma) was used as a standard. Absorbance was measured using a UV/visible spectrophotometer (Amersham Biosciences, Buckinghamshire, UK) at 530 nm.

Acetylcholine esterase activity was determined spectrophotometrically using ACh as a substrate. To measure AChE activity, 300 μL of TRIS buffer (0.1 mol/L, pH 8.0, Sigma), 20 μL of dithiobisnitrobenzoic acid (DTNB, 0.01 mol/L, Sigma), and 10 μL of enzyme suspension were added successively. Acetylcholine (10 μL, 0.1 mol/L, Sigma) was added before the enzymatic reaction was started, and absorbance was monitored using a UV/visible spectrophotometer (Amersham Biosciences) at 405 nm [30].

The MAO activity was assayed by a modification of the procedure of Kalaria et al [31], which is based on the colorimetric determination of the H₂O₂ formed. The reaction mixture (700 μL) contained 100 mmol/L sodium phosphate buffer (pH 7.4), 3 mmol/L sodium azide (Sigma), 100 to 250 μg of tissue protein, and benzylamine at 1 mmol/L (Sigma). The mixture was incubated for 30 minutes at 37°C, and the reaction was stopped by the addition of 0.5 mL of H₂O₂ (Sigma). After 15 seconds, 0.25 mL of 0.75 mol/L hydrochloric acid containing 5% NaDodSO₄ (Sigma) was added to the reaction mixture. Absorbance was measured using a UV/visible spectrophotometer (Amersham Biosciences) at 550 nm. To measure SOD activity [34], serum was diluted with phosphate buffer (pH 7.0, Sigma) by 30 times, and 100 μL of the diluted serum was mixed with 500 μL of distilled water, 200 μL of A buffer (hydroxyl amine [6 mmol/L, Sigma] and hypoxanthine [3 mmol/L, Sigma]), and 200 μL of B buffer (xanthine oxidase [2.6 mmol/L, Sigma] and EDTA [0.1 mmol/L, Sigma]), and the resulting mixture incubated at 37°C for 40 minutes. After incubation, 2 mL of C buffer (sulfanilic acid [3 mmol/L, Sigma] and N-1-naphthylene diamine acid [38 μmol/L, Sigma] in 16.7% of acetic acid [Sigma]) was added to the mixture and incubated at room temperature for 20 minutes. Absorbance was measured using a UV/visible spectrophotometer (Amersham Biosciences) at 550 nm. The SOD activity (unit/mg protein) was determined using a standard curve generated with known concentrations of SOD (Sigma).

Catalase activity was measured by the method of Rigo and Rotilio [35]. The serum (40 μL) diluted by 10 times, was added to 500 μL of phosphate buffer (0.13 mmol/L, pH 7.0, Sigma), 660 μL of distilled water, and 1800 μL of H₂O₂ (15 mmol/L, Sigma), and thoroughly mixed. The rate of change in the absorbance at 240 nm for 5 minutes was recorded. Catalase activity was expressed as unit/mg protein.

2.3. Statistical analyses

All data was expressed as means ± SEM values of 7 rats per group with 3 to 4 replicates per sample. Comparisons of the mean values were performed by 1-way analysis of variance followed by the Dunnett t test using GraphPad Instat software (GraphPad, San Diego, Calif). A probability of 5% or less was accepted as statistically significant [36,37].

3. Results and discussion

There was no mortality in the rats that were fed diets supplemented with E prostrata during the experimental period of 6 weeks and no observable changes in animal autonomic or behavioral patterns.

3.1. Acetylcholine content and AChE and MAO-B activity in the brain

During the course of the normal aging process, concentrations of ACh tend to decrease, resulting in the sporadic lapses of short-term memory that many elderly individuals tend to experience from time to time. This normal, nondebilitating decline in memory, referred to as age-associated memory impairment or benign senescent forgetfulness, is called dementia. However, AD is a serious brain disorder, in which levels of ACh can drop by up to 90%. The gradual death of cholinergic brain cells results in a progressive and significant loss of cognitive and behavioral function [38]. Damage to the cholinergic (ACh-producing)
system in the brain has been linked to the memory deficits associated with AD, and so a shortage of ACh in the brain has been associated with AD [39]. Thus, maintaining or keeping some level of ACh in the brain is essential to the prevention or treatment of dementia. In this study, the ACh level in the brain significantly ($P < .05$) increased by 9.6% and 12.1% in E50 and E100 groups, respectively, compared with the untreated control group (Table 2).

Acetylcholine esterase is an enzyme that degrades the neurotransmitter ACh, producing choline and an acetate group. It is mainly found at neuromuscular junctions and cholinergic synapses in the central nervous system, where its activity terminates synaptic transmission. Acetylcholine esterase has a very high catalytic activity—each molecule of AChE degrades about 25 000 molecules of ACh per second. The choline produced by the action of AChE is recycled—it is transported, through reuptake, back into nerve terminals where it is used to synthesize new ACh molecules [6]. The reduction of cortical AChE activity is a common feature of Alzheimer dementia and appears to be related mainly to the impairment of attention. It occurs very early in the course of the disease, even at a predementia stage, and is likely to indicate functional impairment of the synapses or of the axonal transport of ascending cholinergic neurons [40]. An overall decrease of AChE activity in AD brains was observed [7], and reduced AChE was found in autopsy studies of severe AD [8-10] in correlation with dementia severity [11]. In this study, AChE activity in the brains of the rats significantly increased by 13.1% ($P < .05$) and 19.7% ($P < .01$) in the E50 and E100 groups, respectively (Table 2). This may be related to the increased ACh level in the E50 and E100 groups. Although the reason why an increase in AChE does not reduce ACh level is unclear, this observation is in agreement with previously published reports indicating a negative correlation between the levels of ACh and AChE, even though AChE is an enzyme that degrades the neurotransmitter ACh [6,7,11,40].

Monoamine oxidase-B plays an important role in the metabolism of neuroactive and vasoactive amines in the central nervous system and peripheral tissues [41]. Its activity indicates the severity and/or clinical progress of AD [42]. The elimination of beta-amyloid–induced oxidative damage through the inhibition of the NADPH oxidase represents an attractive therapeutic target for the treatment of AD [43]. In this study, MAO-B activity in the brain of the E100 group significantly decreased by 10.5% compared with the control group (Table 2).

Eclipta extracts have a high percentage of saponins and tannins as medicinally active constituents. The concentration of saponin in the butanol fraction of E. prostrata was 4.5% followed by tannins at 1.0%. Other phytochemicals, such as phytosterols, phenols, and flavonoids, in Eclipta are estimated to be present only in very low concentrations [22]. Eclipta extracts have a beneficial effect on memory impairment in mice [23].

These results demonstrated the potential role of the plant Eclipta in the formation of the important neurotransmitter ACh by increasing or maintaining ACh and AChE levels and decreasing MAO-B activity significantly.

### 3.2. Serum superoxide radicals, catalase, and SOD activities

Oxidative damage is observed early in the progression of AD [44,45]. The formation of the superoxide radical ($O_2^•$) in the serum was inhibited by 9.4% in the E100 group compared with the control group (Table 3). The activity of SOD, which blocks peroxynitrite formation by scavenging superoxide radicals, and facilitates neuronal cell death [46], was increased by 9.6% and 11.6% in the serum of the E50

### Table 2
Acetylcholine content and AChE and MAO activity in the brains of CD rats fed Eclipta extract for 6 weeks

<table>
<thead>
<tr>
<th>Groups</th>
<th>Control</th>
<th>E25</th>
<th>E50</th>
<th>E100</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACh (ng/mg protein)</td>
<td>27.2 ± 1.8</td>
<td>28.7 ± 1.9</td>
<td>29.8 ± 0.1&lt;sup&gt;*&lt;/sup&gt;</td>
<td>30.5 ± 1.3&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>AChE (unit/mg protein per minute)</td>
<td>202.3 ± 11.8</td>
<td>221.9 ± 23.9</td>
<td>228.8 ± 13.8&lt;sup&gt;*&lt;/sup&gt;</td>
<td>242.2 ± 21.0&lt;sup&gt;**&lt;/sup&gt;</td>
</tr>
<tr>
<td>MAO (nmol/mg protein per minute)</td>
<td>2.2 ± 0.1</td>
<td>2.1 ± 0.1</td>
<td>2.0 ± 0.1</td>
<td>1.9 ± 0.1&lt;sup&gt;**&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

All data are expressed as means ± SEM values of 7 rats per group with 3 to 4 replicates per sample. Comparisons of the mean values were performed by 1-way analysis of variance followed by the Dunnett $t$ test. A probability of 5% or less was accepted as statistically significant.

<sup>*</sup> $P < .05$ significant difference.

<sup>**</sup> $P < .01$ significant difference.

### Table 3
Serum superoxide radical level and catalase activity of CD rats fed Eclipta extract for 6 weeks

<table>
<thead>
<tr>
<th>Groups</th>
<th>Control</th>
<th>E25</th>
<th>E50</th>
<th>E100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superoxide radicals (nmol/mg protein)</td>
<td>139.4 ± 13.2</td>
<td>136.4 ± 12.2</td>
<td>127.9 ± 13.1</td>
<td>126.2 ± 10.8&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>Catalase activity (μmol min&lt;sup&gt;−1&lt;/sup&gt; mL&lt;sup&gt;−1&lt;/sup&gt;)</td>
<td>128.3 ± 10.2</td>
<td>129.3 ± 10.6</td>
<td>134.9 ± 12.6</td>
<td>138.0 ± 10.6</td>
</tr>
</tbody>
</table>

All data are expressed as means ± SEM values of 7 rats per group with 3 to 4 replicates per sample. Comparisons of the mean values were performed by 1-way analysis of variance followed by the Dunnett $t$ test. A probability of 5% or less was accepted as statistically significant.

<sup>*</sup> $P < .05$ significant difference.
and E100 groups, respectively, compared with the control group (Fig. 1). Catalase activity was increased in the E50 and E100 groups. However, no significant difference was found in the catalase activity among experimental groups (Table 3).

The reduced formation of superoxide radicals may be explained by a report in which AChE hydrolyzed lipid peroxides and protected low density lipoprotein (LDL) against oxidation [47]. It is reported that manganese SOD overexpression reduced the oxidative stress and memory deficiency in a transgenic mouse model of AD [48]. Taken together, this data suggests that Eclipta may play an important protective role in the pathogenesis of oxidative damage caused by AD.

The oral treatment of mildly hypertensive male subjects aged 40 to 55 years with encapsulated Eclipta powder (3 g/d) alleviated complications due to oxidative stress [49]. Eclipta extracts significantly increased antioxidant activity in in vitro systems [24]. Recent studies suggest that oxidative damage due to free radicals may be an important factor in neurological disorders [50,51]. Present results suggest that the memory-enhancing effects of Eclipta may be associated with increased ACh and decreased oxidative stress and so may improve brain function.

From these results, we conclude that E prostrata increases the formation of ACh and inhibits oxidative stress in rats. The beneficial effects of E prostrata on brain health, which we demonstrate in this study in a rat model, may have implications for developing strategies that will improve antiamnesia and memory functions of the brain in humans. However, further studies are needed to verify the detailed mechanism and its possible function in humans.

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References


