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트리메틸틴 처리로 유도된 기억 · 학습 능력 손상 모델에 대한 계피와 금앵자 혼합추출물의 개선 효과

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Ameliorating Effects of *Cinnamomum loureiroi* and *Rosa laevigata* Extracts Mixture against Trimethyltin-induced Learning and Memory Impairment Model

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ABSTRACT

Background: A critical features of Alzheimer's disease (AD) is cognitive dysfunction, which partly arises from decreased in acetylcholine levels. AD afftected brains are characterized by extensive oxidative stress, which is thought to be primarily induced by the amyloid beta (Aβ) peptide. In a previous study, *Cinnamomum loureiroi* tincture inhibited acetylcholinesterase (AchE) activity. That study identified AChE inhibitor in the *C. loureiroi* extract. Furthermore, the *C. loureiroi* extract enhanced memory in a trimethyltin (TMT)-induced model of cognitive dysfunction, as assessed via two behavioral tests. *Rosa laevigata* extract protected against oxidative stress-induced cytotoxicity. Administrating *R. laevigata* extracts to mice significantly reversed Aβ-induced learning and memory impairment, as shown in behavioral tests.
Methods and Results: We conducted behavioral to examine the synergistic effects of *C. loureiroi* and *R. laevigata* extracts in inhibiting AChE and counteracting TMT-induced learning and memory losses. We also performed biochemical assays. The biochemical results showed a relationship between increased oxidative stress and cholinergic neurons damage in TMT-treated mice.
Conclusions: A diet containing *C. loureiroi* and *R. laevigata* extracts ameliorated learning and memory impairments in the Y-maze and passive avoidance tests, and exerted synergistic inhibitory effect against AChE and lipid peroxidation.

***Key Words*:** *Cinnamomum loureiroi, Rosa laevigata,* Alzheimer's Disease, Learning, Memory

INTRODUCTION

Alzheimer's disease (AD) was originally described in 1906 by Alois Alzheimer based on the observation of amyloid plaques and neurofibrillary tangles during an autopsy of a patient. The primary clinical feature of AD is cognitive dysfunction, which has been demonstrated to be closely related to the loss of the cholinergic function (Berchtold *et al*., 1998).

It has been frequently reported that AD patients display a decrease in acetylcholine (ACh) levels within the basal forebrain (McGleenon *et al*., 1999). ACh is an important neurotransmitter that plays a critical role in learning and memory processes. In the central cholinergic systems, ACh is synthesized from choline and acetyl-coenzyme A by choline acetyltransferase (ChAT) (Ohno *et al*., 2001).

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ChAT and acetylcholinesterase (AChE) are responsible for the synthesis and hydrolysis of ACh in cholinergic neurons, respectively. AChE inhibitors that inhibit the hydrolysis of ACh to increase the levels of cholinergic neurotransmitters are widely administered to patients with AD. Synthetic AChE inhibitors, including tacrine, donepezil, and rivastigmine, have been tested clinically. However, the side effects of these drugs have led to the development of new AChE inhibitors from nontoxic natural resources.

Cinnamomum loureiroi contains large amounts of bioactive molecules, including essential oils, tannin, mucus, and coumarins. *C. loureiroi* is one of the most widely used herbal medicines in Korea, belongs to the family *Lauraceae*, and is often used as a spice and flavoring agent.

Trimethyltin (TMT) is a compound that induces neuronal degeneration in the hippocampus, and results in behavioral alternations including cognitive impairments. TMT has been widely used as a neurotoxin because of its effects associated with *in vivo* behavioral changes and biochemicaldeficits in the brains (Choi *et al*., 2016).

Our study have previously demonstrated that the *C. loureiroi* extract significantly inhibited AChE *in vitro* and was neuroprotective against TMT-induced cognitive dysfunction in mice (Kim *et al*., 2016). We further identified 2,4-bis-(1,1-dimethylethyl) phenol from *C. loureiroi* as a potential AChE inhibitor.

AD brains are characterized by extensive oxidative stress, which is believed to be primarily induced by the amyloid beta (Aβ) peptide. Many investigators surmise that deposition of Aβ peptide is the crucial step in AD pathogenesis. Aβ overproduction leads to the intracellular accumulation of reactive oxygen species (ROS), which eventually results in the peroxidation of the cells, modification of proteins, DNA/RNA damage, and cell death (Butterfield *et al*., 2007).

Rosa laevigata, a plant of the *Rosaceae* family, is native to China and Japan. It tastes sour and sweet and passes to kidney, urinary bladder, and large intestine channels. *R. laevigata* relieves nocturnal emission and leucorrhagia and reduces the frequency of urination.

We have previously investigated the protective effects of *R. laevigata* aginst Aβ-induced oxidative stress and learning and memory impairment in mice. The *R. laevigata* extract exerted an anti-amnestic activity *in vivo* through the blockade of Aβ-induced neuronal damage. Our previous findings also indicated that the active component of *R. laevigata* is the 1,2-benzenedicarboxylic acid, dinonyl ester (Choi *et al*., 2006).

AChE is a potent amyloid-promoting factor when compared with other Aβ-associated proteins. Thus, in addition to its role in the cholinergic synapses, AChE may function by accelerating Aβ formation and could play a role during amyloid deposition in AD. Previous studies suggested that Aβ injection directly inhibits various cholinergic neuronal functions. These previous results specifically indicated the potential role of free radical toxicity and the damage of cholinergic neurons, demonstrating an increase in oxidative stress and AChE activity (Nabeshima and Nitta, 1994; Choi *et al*., 2009). These results indicated an interaction between AChE and Aβ.

In this present study, to test the potential effects of the combination of the AChE inhibitory effect of *C. loureiroi* and the anti-oxidative effect of *R. laevigata*. The possible protective effects of *C. loureiroi* and *R. laevigata* extracts against TMT-induced neuronal damage were examined.

To investigate the effect of the extracts on learning and memory impairment in mice, *in vivo* behavioral and biochemical assays were performed. The present biochemical results demonstrated a relationship between increased oxidative stress and damaged cholinergic neurons in TMT-treated mice. The damage induced by TMT injection was potently reversed by the *C. loureiroi* and *R. laevigata* extracts.

MATERIALS AND METHODS

1. Materials

Acetylthiocholine iodide, 5,5′-dithiobis-(2-nitro)benzoic acid (DTNB), dimethyl sulfoxide (DMSO), and TMT were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were of analytical grade unless otherwise specified.

2. Sample preparation

Dried *Cinnamomum loureiroi* and *Rosa laevigata* were purchased from Gyung-dong market, an oriental medicine store in Seoul, South Korea. The plant materials were authenticated by the Institute of Biotechnology at Korea University.

The dried *C. loureiroi* and *R. laevigata* were ground

into a fine powder form and subsequently extracted with 10 volumes of 70% ethanol. The samples were placed on a shaker over 12 h at 1.57 × g. The extract was filtered through a No. 42 filter paper (Whatman Co., Maidstone, England). The resulting ethanol extracts were evaporated using a rotary evaporator (Eyela, Tokyo Rikakikai Co., Ltd., Tokyo, Japan) under reduced pressure at 37℃ (Choi *et al*., 2016).

3. Cell culture

The PC12 cell line (CRL-1721) was obtained from the American Type Culture Collection (Manassas, VA, USA). The PC12 cells were cultured in RPMI 1640 medium supplemented with 10% (v/v) horse serum from a donor herd, 5% (v/v) fetal bovine serum, and 1% (v/v) antibiotic-antimycotic (Gibco BRL, Gaithersburg, MD, USA). The cells were cultured on 100 ㎜ diameter tissue culture dishes (BD Biosciences, Franklin Lakes, NJ, USA) and maintained in an incubator (37℃, water saturated, and 5% CO_2 atmosphere). The cells were sub-cultured when a confluency of 80 - 90% (split ratio 1 : 4) was attained. The medium was refreshed approximately three times a week (Choi *et al*., 2013).

4. Measurement of AChE activity

The AChE activity was determined using the modified spectrophotometric method of Ellman. ACh iodide was used as the reaction substrate, and DTNB was used to measure AChE activity (Ellman *et al*., 1961; Kim *et al*., 2011).

Briefly, for the enzyme preparation, PC12 cells were homogenized using a homogenizer (Glas-Col, Terre Haute, IN, USA) with Tris-hydrochloride (HCl) buffer (20 mM Tris-HCl (pH 7.5) containing 150 mM sodium chloride (NaCl), 10 mM magnesium chloride (MgCl), and 0.5% Triton X-100). After homogenization, the samples were centrifuged at 10,000 × g for 15 min and the resulting supernatants were used as the enzyme source.

The protein concentration was determined using a protein quantification assay kit (Bio-Rad, Hercules, CA, USA), with bovine serum albumin as the protein standard. 10 ㎕ of each sample were mixed with 10 ㎕ of enzyme solution, and were subsequently added to 70 ㎕ of the reaction mixture [50 mM sodium phosphate buffer (pH 8.0) containing 0.5 mM ACh iodide and 1 mM DTNB], and incubated at 37℃ for 15 min. The ACh iodide and enzyme reaction were monitored at a wavelength of 405 ㎚ using a 96-well micro plate reader (GENios, Tecan Ltd., Männedorf, Switzerland).

5. Animals

Institute of Cancer Research (ICR) mice (5-week-old males) were purchased from DBL (Eumseong, Korea) and were provided access to food and water *ad libitum*. The *C. loureiroi* and *R. laevigata* extracts were mixed into the feed. Subsequently, TMT, which was dissolved in an NaCl solution, was administered through intraperitoneal (IP) injection. The control group was injected with NaCl without TMT.

All sample groups were injected with TMT (100 ㎕ per mouse; 2.5 ㎎/㎏ body weight) (Choi *et al*., 2016). However, the animals of each group were maintained on diets differentially supplemented with *C. loureiroi* and *R. laevigata* extracts (30 or 50 ㎎/㎏ per day, respectively) for 4 weeks. All experimental procedures abided by the guidelines established by the Animal Care and Use Committee of Korea University (KUIACUC-2017-142).

6. Y-maze test

The Y-maze test was performed 2 days after the TMT injection. The maze consisted of black plastic, with three maze arms measuring each 33 ㎝ long, 15 ㎝ high, 10 ㎝ wide, and separated by an angle of 120°. Each mouse was placed at the end of one arm and allowed to move freely through the maze during an 8-min period. The sequence of arm entries was recorded manually.

Spontaneous alternation behavior was defined as entry into all three arms by consecutive choice in overlapping triplet sets. The percentage of spontaneous alternation behavior was calculated as the ratio of actual-to-possible alternations (Kim *et al*., 2017).

$$\text{Alternation behavior (\%)} = (\text{possible alternations}) \times 100 \,/\, (\text{total number of arm entries} - 2)$$

7. Passive avoidance test

The passive avoidance test was performed 2 days after the TMT injection. The apparatus consisted of an illuminated chamber connected to a dark chamber.

An acquisition trial was performed on day 1. Each

mouse was placed in the apparatus and was left there for 1 min with no light or shock, followed by a 2 min period with light and no shock, to habituate to the apparatus. Subsequently, the mice were individually placed in the illuminated chamber. Immediately after entering the dark chamber, an inescapable scrambled electric shock (0.5 ㎃ for 1 s) was delivered through the floor grid (acquisition trial). The mice were subsequently returned to their cages. Each mouse was placed again in the illuminated chamber 24 h later (retention trial).

The interval between placement in the illuminated chamber and entry into the dark chamber was measured as the latency. The maximum testing limit for step-through latency was 300 s (Kim *et al*., 2017).

8. Quantification of ACh content in mice brain tissues

The ACh brain content was measured using the method of Hestrin as described previously based on the reaction of ACh and hydroxylamine (Hestrin, 1949).

Mice were sacrificed, and their brains were dissected and maintained at −80℃ prior to use. The brains were homogenized in cold phosphate buffered saline (PBS) in an ice bath. The homogenates were directly centrifuged twice at a 30 s interval at 33,600 × g for 10 s. Subsequently, 1 ㎖ of the homogenate was mixed with 2 ㎖ alkaline hydroxylamine reagent. After at least 1 min, the pH was adjusted to 1.2 ± 0.2 with 1 ㎖ HCl solution and 1 ㎖ iron solution. The density of the purple brown color was determined at 540 ㎚.

9. Determination of lipid peroxidation in mice brain tissues

The brains were homogenized in cold PBS in an ice bath. The homogenates were directly centrifuged twice at a 30 s interval at 33,600 × g for 10 s. Aliquots of the supernatant were used to determine the malondialdehyde (MDA) levels and protein content in the brain.

The MDA level was assayed for lipid peroxidation products by using previously method (Choi *et al*., 2009). Briefly, 80 ㎕ of each homogenate were mixed with 480 ㎕ phosphoric acid (1%, v/v) followed by the addition of 160 ㎕ thiobarbituric acid solution (0.67%, w/v). The mixture was incubated at 95℃ in a water bath for 45 min. After cooling, the colored complex was extracted with n-butanol. The butanol phase was separated by centrifugation, and the absorbance was measured using tetramethoxypropane as a standard at 532 ㎚.

10. Statistical analysis

All data were expressed as means ± standard deviation (SD). The data were analyzed using Duncan's Multiple Range Tests (DMRT) in statistical analysis system (SAS Institute Inc., Cary, NC, USA). The statistical significance of differences among groups was calculated by One-way (ANOVA). The statistical difference was set at $p < 0.01$.

RESULTS AND DISCUSSION

The cholinergic system impairment observed in patients with AD leads to the cognitive and behavioral dysfunctions commonly associated with dementia. The cholinergic deficit in patients with AD is demonstrated by reduced ChAT activity and increased AChE activity. Based on these previous findings, there has been increasing interest in developing AChE inhibitors for therapeutic use in AD. AChE inhibitors decrease the hydrolysis of ACh in the brain, thereby boosting cholinergic neurotransmission. This effect was based on the premise that boosting ACh levels would attenuate the neuronal deficits and cognitive impairments present in patients with AD (McGleenon *et al*., 1999).

Aβ is known to increase oxidative stress in nerve cells. This oxidative stress is mediated by ROS, which are generated continuously in the nervous tissue during normal metabolism and neuronal activity. When the metabolic balance between ROS and the antioxidant system is lost, oxidative stress occurs. Free radicals, including hydrogen peroxide, are generated by multiple enzymatic and nonenzymatic pathways, leading to the initiation of the oxidative stress cascade (Smith *et al*., 2007; Jung *et al*., 2012; Ko and Lee, 2015).

Previous reports indicated that AChE accelerates amyloid formation, and is a potent amyloid-promoting factor when compared with other Aβ-associated proteins. Thus, AChE may function by accelerating Aβ formation and could play a role during amyloid deposition in AD (Inestrosa *et al*., 1996). In our previous study, we demonstrated that *Cinnamomum loureiroi* extract significantly inhibited AChE *in vitro* and was neuroprotective against TMT-induced

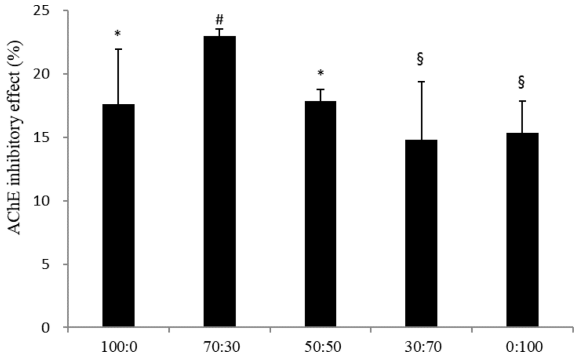

Fig. 1. Acetylcholinesterase (AChE) inhibition by *C. loureiroi and R. laevigata* extracts. The sample groups were treated with various mixtures of *C. loureiroi and R. laevigata* extracts (100 : 0, 70 : 30, 50 : 50, 30 : 70, 0 : 100, w/w). The concentration of all samples was 1 ㎎/㎖. Each value represents the means ± SD (n = 4), $p < 0.01$. Different superscript symbols (*, §, and #) represent

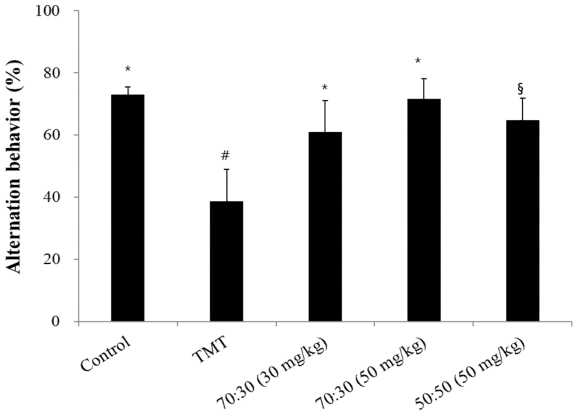

Fig. 2. The effect of *C. loureiroi and R. laevigata* extracts on TMT-induced memory deficit in mice as measured by a Y-maze test. Spontaneous alternation behavior was measured over 8 min. The control group was injected with a sodium chloride solution, while the TMT group was injected with a sodium chloride solution containing TMT. Sample groups were injected with TMT solution after pretreatment with *C. loureiroi and R. laevigata* extracts (70 : 30, 30, or 50 ㎎/㎏ of body weight per day; 50 : 50, 50 ㎎/㎏ of body weight per day, respectively). Each value represents the means ± SD (n = 8), $p < 0.01$. Different superscript symbols (*, §, and #) represent statistical differences between groups.

cognitive dysfunction in mice. The *Rosa laevigata* extract showed anti-amnestic activity *in vivo* through the blockade of Aβ-induced neuronal damage. In this study, the possible synergistic protective effect of *C. loureiroi* and *R. laevigata* extracts against TMT-induced neuronal damage was examined.

In order to confirm the synergistic effect of *C. loureiroi* and *R. laevigata* extracts, the AChE inhibitory effect was evaluated using Ellman's method. As shown in Fig. 1, mixtures of various ratios of the extracts were tested and the 70 : 30 ratio resulted in the highest effect (22%). Our study previously reported that the extract of *C. loureiroi* significantly inhibited AChE, and identified 2,4-bis(1,1-dimethylethyl)phenol from *C. loureiroi* as a potential AChE inhibitor. Also 1,2-benzenedicarboxylic acid dinonyl ester from *R. laevigata* demonstrated AChE inhibitory effect. It is believed that the AChE inhibitory effect has been shown in these components.

This study demonstrated that *C. loureiroi* and *R. laevigata* extracts inhibited AChE *in vitro*, and that the 70 : 30 mixture resulted in a improved effect when compared with other mixtures. *In vivo* experiment, compared 50 : 50 and 70 : 30 which showed the best effect *in vitro* experiment. To determine the anti-amnestic effects of *C. loureiroi* and *R. laevigata* extracts combination *in vivo*, a Y-maze test was performed using TMT-treated mice.

TMT is considered a useful tool to induce neurodegeneration *in vivo*. Animals exposed to TMT develop behavioral alterations featuring cognitive impairments. Furthermore, TMT induces selective neuronal cell death (Geloso *et al*., 2011).

Consistently with previous reports, our study confirmed that exposure to TMT impaired learning and memory in mice. The Y-maze test was performed 3 days after the TMT injection, and resulted in significant impairments of the spatial working memory (34% decrease in the Y-maze alternation behavior) in TMT-treated mice compared with the control group. However, the TMT-induced decrease in working memory was reversed to the control level by pretreatment with the 70 : 30 extract mixture, namely 30 ㎎/㎏ and 50 ㎎/㎏ of *C. loureiroi* and *R. laevigata* extracts, respectively, per day (Fig. 2).

This improvement was similar to the 400 ㎎/㎏ per day concentration of *C. loureiroi* ethanol extract in our previous study (Kim *et al*., 2016). The number of arm entries did not change among any of the experimental groups (data not shown), indicating an intact general locomotion following the TMT injection or the sample diet. These results suggested that the behavioral changes observed were specific to the extract-associated attenuation

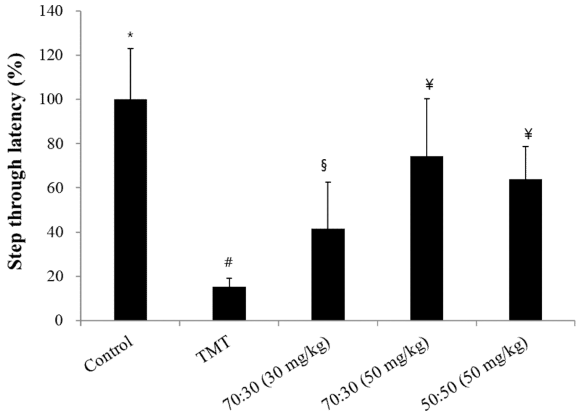

Fig. 3. The effect of the *C. loureiroi and R. laevigata* extracts on TMT-induced memory deficits in mice as assessed with a passive avoidance test. Step-through latency was measured over 5 min. The control group was injected with a sodium chloride solution. The TMT group was injected with a sodium chloride solution containing TMT. Sample groups were injected with TMT solution after pretreatment with *C. loureiroi and R. laevigata* (70 : 30, 30, or 50 ㎎/㎏ of body weight per day; 50 : 50, 50 ㎎/㎏ of body weight per day, respectively). Each value represents the means ± SD ($n = 8$), $p < 0.01$. Different superscript symbols (*, #, §, and ¥) represent statistical differences between groups.

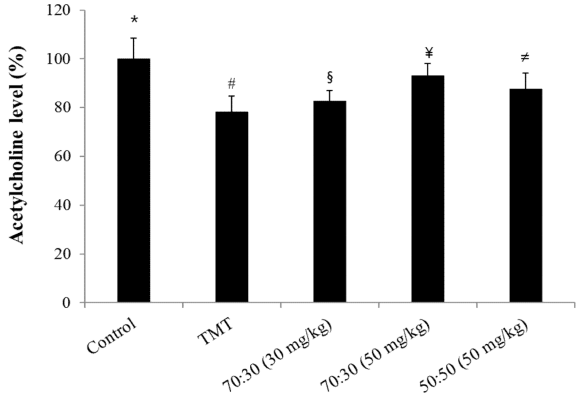

Fig. 4. Effect of *C. loureiroi and R. laevigata* extracts on acetylcholine content in mice brains treated with TMT. The control group was injected with a sodium chloride solution. The TMT group was injected with a sodium chloride solution containing TMT. Sample groups were injected with TMT solution after pretreatment with *C. loureiroi and R. laevigata* extracts (70 : 30, 30 ㎎/㎏ or 50 ㎎/㎏ of body weight per day; 50 : 50, 50 ㎎/㎏ of body weight per day, respectively). Each value represents the means ± SD ($n = 8$). $p < 0.01$. Different superscript symbols (*, #, §, ¥, and ≠) represent statistical differences between groups.

in spatial memory.

The passive avoidance test was performed 3 days after the TMT injection, and resulted in a significant reduction (80% decrease) in step-through latency in the TMT-treated mice when compared with the control group. Administering a diet consisting of the *C. loureiroi* and *R. laevigata* extracts attenuated the TMT-induced impairment in the passive avoidance test (Fig. 3).

Our findings suggested that a diet containing the extracts of *C. loureiroi* and *R. laevigata* had a protective effect against learning and memory deficits induced by TMT. Furthermore, the 70 : 30 mixture indicated a dose-dependent attenuation of TMT-induced impairment in the Y-maze and passive avoidance test. Thus, the extract displayed a significant anti-amnestic effect in our mouse model.

Based on these results, it is possible that the AChE inhibitory effects of the extracts combination may account for the anti-amnestic effects observed in this study. The 70 : 30 concentration showed a statistically significant increase *in vitro* experiments. Although the activity effect did not seem to be much, it showed a significant effect even at low concentrations in Y-maze test. Following the behavioral tests, the mice serum was collected to evaluate the acute toxicity of *C. loureiroi* and *R. laevigata* extracts mixtures using the serum transaminase reagents kit. Serum aminotransferases were not significantly different among the experimental groups, indicating a lack of liver toxicity (data not shown).

The ACh content in the brains of the experimental groups was determined by spectrophotometric analysis. As shown in Fig. 4, ACh was significantly decreased in the TMT group (about 20%). However, the ACh levels were increased to the level of the controls following treatment with *C. loureiroi* and *R. laevigata* extracts. The sample groups which were administered the 70 : 30 (30 ㎎/㎏, 50 ㎎/㎏) and 50 : 50 (50 ㎎/㎏) diet mixtures recovered almost to the same level as the control group.

One of the mechanisms of TMT-induced neurodegeneration is the increase in AChE activity. These changes were correlated with the degeneration of cholinergic axons and changes in ACh levels and their receptors. Indeed, TMT toxicity has been reported to cause a decrease of ACh release in cerebral tissues (Loullis *et al*., 1985).

As shown in Fig. 2 and 3, the administration of *C. loureiroi* and *R. laevigata* extracts in the diet of TMT-

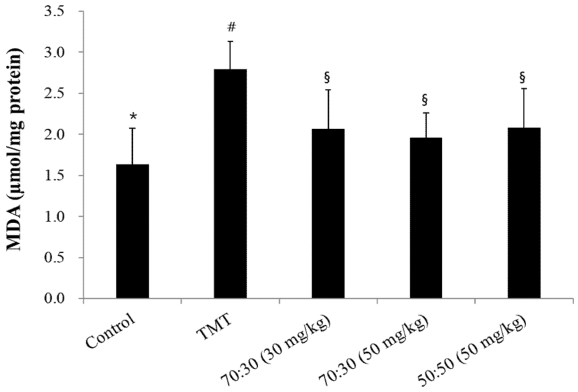

Fig. 5. The effect of *C. loureiroi and R. laevigata* extracts on lipid peroxidation in mice brains. The control group was injected with a sodium chloride solution. The TMT)group was injected with a sodium chloride solution containing TMT. Sample groups were injected with TMT solution after pretreatment with *C. loureiroi and R. laevigata* extracts (70 : 30, 30, or 50 ㎎/㎏ of body weight per day; 50 : 50, 50 ㎎/㎏ of body weight per day). Each value represents the means ± SD (n = 8). $p < 0.01$. Different superscript symbols (*, #, and §) represent statistical differences between groups.

treated mice attenuated TMT-induced learning and memory impairments. The group administered *C. loureiroi* and *R. laevigata* extracts exhibited increased ACh levels compared with the levels in the TMT alone group. Thus, *C. loureiroi* and *R. laevigata* extracts possibly improved the learning and memory ability in TMT-treated mice by inhibiting AChE and consequently restoring ACh levels.

Another possible mechanism is that TMT-induced neuronal damage generates ROS. Previous reports demonstrated that TMT promotes the formation of a major product of lipid peroxidation (Wang *et al*., 2008; Shuto *et al*., 2009). To determine the TMT-induced lipid peroxidation in the mice brains, MDA was measured after the behavioral tests. The MDA levels increased significantly (1.16 μM/㎎ protein) in the TMT-injected group when compared with those of the control group. The increase in MDA levels indicated an elevation of lipid peroxidation in the brains of TMT-injected mice. Diets containing *C. loureiroi* and *R. laevigata* extracts reversed the effects of TMT-induced lipid peroxidation (Fig. 5). Although 70 : 30 (30 ㎎/㎏, 50 ㎎/㎏) are not statistically different from 50 : 50 (50 ㎎/㎏), it is a meaningful result to show its effect even at low concentrations (30 ㎎/㎏).

These findings indicated that these changes may be the mechanisms underlying the improvement of learning and memory ability in mice by attenuating AChE and oxidative stress. The pretreatment with the *C. loureiroi* and *R. laevigata* extracts mixture reversed the ACh reduction and MDA elevation in mice treated with TMT. Taken together, our present findings indicated that *C. loureiroi*and *R. laevigata* extracts mixture inhibited TMT-induced neuronal cell damage and improved TMT-induced learning and memory deficits.

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