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Original Research Article

Effects of Aqueous Extract of Unpolished Dark Purple Glutinous Rice, Var Luem Pua, on ROS in SK-N-SH Cells and Scopolamine-induced Memory Deficit in Mice

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Abstract

Purpose: *To investigate the antioxidative and memory-enhancing effects of aqueous extract of unpolished Thai rice strain of Luem Pua (LP) in SK-N-SH cells and scopolamine-induced memory deficit in mice.*
Methods: *In SK-N-SH cells, viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay while intracellular reactive oxygen species (ROS) levels were quantified after pretreatment with LP (0, 200, 600 and 1000 µg/mL) in the presence of hydrogen peroxide (H_2O_2). In mice, memory was impaired by injecting 2 mg/kg/day scopolamine, for 18 consecutive days. On each day, mice were also force-fed with LP 0, 90 or 180 mg/kg. On the last 5 days of treatment, memory was tested using passive avoidance (PA) and Morris water maze (MWM) tests.*
Results: *At concentration up to 1,000 µg/mL LP had no effect on SK-N-SH cell viability and significantly reduce intracellular ROS levels of SK-N-SH cells with or without H_2O_2. Mice that received 90 or 180 mg/kg LP showed a significant decrease in latency time in PA test and an increase in escape latency time in MWM test. These data suggest that LP antagonizes the effect of scopolamine on memory.*
Conclusion: *LP extract has anti-oxidative and memory-enhancing effects in cell culture and mice. The rice may be a nutraceutical helpful for promoting brain health.*

Keywords: *Dark purple rice, Leum Pua rice, Reactive oxygen species, Memory, Scopolamine, Cell culture*

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INTRODUCTION

Dementia has a wide range of symptoms associated with a decline in memory or other thinking skills which could reduce a person's ability to perform everyday activities. The most common dementia type is Alzheimer's disease (AD) which accounts for 60 to 80 percent of cases and affects approximately 10 - 15 % of people older than 65 years [1]. At present, the definite causes of AD remain unknown, and the treatment remains symptomatic without an effective cure. A decrease in acetylcholine in the brains of patients with AD, however, appears to be a critical element in producing dementia [2] and the cholinergic neuronal system is the major

therapeutic rational behind the use of nootropic agents. Scopolamine, a muscarinic cholinergic receptor antagonist, has been widely adopted for studying cognitive deficits in experimental animals [3]. In addition, over the past decade, much attention has been focused on oxidative stress as one of the important mechanisms in AD pathogenesis. Reactive oxygen species (ROS) accumulate in the cells leading to oxidative stress, causing damage to DNA, lipids, proteins and mitochondria that ultimately leads to neuronal death [4,5].

Over the last few decades the influence of diet in protecting neurons against neurodegenerative diseases and enhance cognitive abilities is widely accepted [6]. Recently, previous studies indicate that flavonoid-rich foods such as *Camellia sinensis* (tea) [7,8], *Gingko biloba* [9-11], *Theobroma cacao* (cocoa) [12-14], and *Vaccinium* spp. (blueberry) [15-17] improve learning and memory in both young and old animals, and humans [18].

Rice (*Oryza sativa*) is the most widely consumed staple food for a large part of the world's human population, especially in Asia. Among many varieties of rice, pigmented rice is considered an enriched source of bioflavonoids, and is widely used for therapeutic purposes in traditional and folk medicine. Luem Pua is one of the aromatic and indigenous purple sticky rice, enriched with flavonoids, especially anthocyanins, and have total antioxidant higher than other black rices [19], The present study was aimed to examine the effects of the aqueous extract of Luem Pua rice (LP) in reducing the ROS in SK-N-SH cell cultures and on learning and memory of the animals that were impaired by scopolamine chronic treatment.

EXPERIMENTAL

Preparation of Leum Pua rice extract (LP)

The unpolished Thai rice strain of Luem Pua came from Phitsanulok Rice Research Center, Phitsanulok province, Thailand. One kilogram of the rice was crushed into powder and soaked in 1.0 L distilled water for 24 h at room temperature with intermittent stirring. After filtration, the same volume of n-butanol was added to the aqueous solution. The crude extract was obtained by removal of both solvents using a vacuum evaporator at 50 ºC followed by a high vacuum pumping system. The percent yield of the rice crude extract was 1.97. It was stored in cool tight container and protected from light until required.

Evaluation of cell culture and cell viability

SK-N-SH human neuroblastoma cells (HTB-11) (ATCC; Manassas, VA) were used in this study and cultured in minimum essential medium (MEM) with supplemented nutrients and essential conditions (GIBCO, Invitrogen Corporation; Grand Island, NY). Cell viability was evaluated by spectrophotometric analysis using MTT (3-(4,5-dimethylthiazolyl-2-yl)-2,5-diphenyl-tetrazolium bromide) (Sigma-Aldrich; St. Louis, MO). LP was diluted in MEM, added to the cells at final concentrations 0, 600, 800 and 1,000 µg/mL, and incubated at 37 °C for 24 h after which the MTT solution was added to each well at a final concentration of 500 µg/ml. Formazan crystals formed by the living cells were then dissolved in isopropanol and measured at 570 nm by Multi-Detection microplate reader (BioTek Instruments, Inc.; Winooski, VT).

Determination of intracellular reactive oxygen species (ROS) levels

Intracellular ROS levels were quantified using 2′, 7′-dichlorodihydrofluorescein diacetate (DCFH-DA) (Sigma-Aldrich; St. Louis, MO) as previously described [20] with some adaptation. In brief, SKN-SH cells (2 x 10^5 cells/mL) were incubated with 50 µM DCFH-DA at 37 °C in the dark for 45 min, followed by 45 min incubation with 200, 600 or 1,000 µg/mL LP (diluted in MEM). The cells were then treated with 300 or 600 µM H_2O_2 (Merck Schuchardt OHG, Hohenbrunn, Germany) for 30 min. The fluorescence intensity of DCF was quantified by a Multi-Detection microplate reader at 485 nm excitation and 530 nm emission wavelengths.

Scopolamine-induced memory deficits in mice

Male ICR mice (6 - 8 weeks old, 25–35 g body weight) were purchased from the National Laboratory Animal Center, Mahidol University, Bangkok, Thailand. The animals were kept in an air-conditioned room maintained at 25 ± 2 ºC with a 12:12 h light: dark cycle, with feed and water *ad libitum*. All animal experiments were performed in accordance with the guidelines of the Animal Ethics Committee, Faculty of Medicine, Khon Kean University, Thailand, record number AEKKU 13/2555 reference number 0514.1.12.2/8.

The animals were divided into 6 groups, 8-10 mice per group. The treatment duration was 18 days. On each day, mice received 2 treatments. Firstly, the animals were force-fed either with distilled water or LP (90 or 180 mg/kg). Thirty min

later, either normal saline or 2 mg/kg scopolamine were injected intraperitoneally (i.p). Passive avoidance (PA) and Morris water maze (MWM) tests were done on the last 5 days of treatment.

Passive avoidance test

Passive avoidance, a fear-aggravated test used to evaluate learning and memory in rodents, was done 15 min after the animal received a second treatment on days 14 (training day) and 15 (test day) of treatment. The details of the test have been described earlier [20]. In brief, the apparatus consists of two distinct compartments, light and dark compartments separated by a sliding door. On training day, mouse was placed in the light compartment and allowed to explore for 30 s, at which point the door was opened to allow the mouse enter the dark compartment, and then the door was closed and an electrical foot shock (0.25 mA) was delivered for 2 s, after that the mouse was removed to its home cage. Test day (24 h after training), the mouse was tested by same protocol as on training day except electrical foot shock was not delivered when the animal entered the dark compartment. The time taken for a mouse to enter the dark compartment after door opening was defined as latency time. Latency time was recorded up to 300 s.

Morris water maze test

The Morris water maze, a test of spatial learning for rodents, was used at 15 min after the normal saline or 2 mg/kg scopolamine injection on days 16, 17 and 18 of treatment [20]. In brief, the Morris water maze consisted of a milky water-filled circular plastic pool 66 cm in diameter which was divided into four quadrants of equal areas. A circular platform submerged below the water surface was placed in the center of one quadrant (the target quadrant).

A mouse was trained by placing it on the platform for 1 min then it was put into the water facing the edge of the pool in the quadrant opposite to the target quadrant, and allowed the maximal time of 1 min for the animal to swim and locate the platform. The time used to locate the platform was expressed as escape latency. If the animal failed to find the platform in 60 s, it was manually guided to the platform and placed on the platform for 15 s. On each day, the tests were performed 3 times with an inter-trial interval of 15 min [21].

Statistical analysis

Data were assessed by one-way analysis of variance (ANOVA) and all pairwise multiple comparison procedures (Fisher's LSD method). A *p*-value less than 0.05 were considered as statistically significant.

RESULTS

***In vitro* cytotoxicity test and the effects on ROS of LP in SK-N-SH cells**

The results showed that LP, at concentrations up to 1000 µg/ml, did not affect SK-N-SH cell viability. In addition, the numbers of viable cells were mildly increased after the exposure to LP at 800 and 1,000 µg/mL (Figure 1).

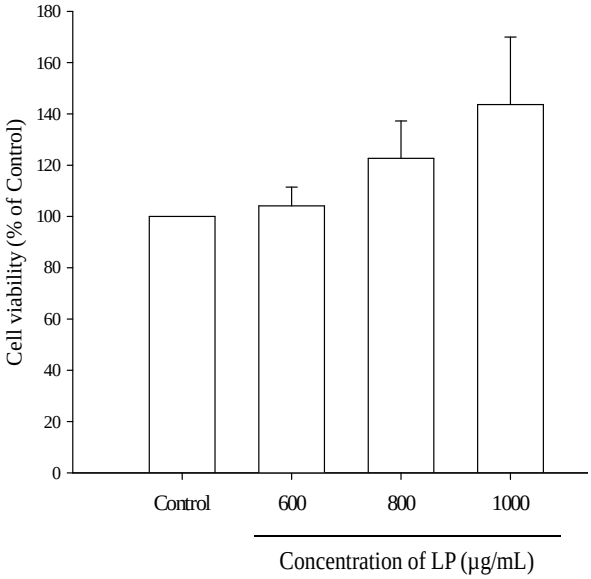

Figure 1: Cytotoxicity screening of Luem Pua rice extract (LP) in SK-N-SH cells. SK-N-SH cells were treated with LP at the concentrations of 600, 800 or 1,000 µg/mL. After 24-hours of incubation, cytotoxicity was determined by the MTT assay. Data are presented as mean ± S.E.M.

The cellular oxidative stress was evaluated by measurement of intracellular ROS levels. As shown in Figure 2A, the exposure of SK-N-SH cells to LP 200, 600 and 1000 µg/mL led to a significant decrease of intracellular ROS levels when compared to the untreated cells. Pre-incubation of SK-N-SH cells with LP at 200, 600 or 1000 µg/mL significantly decreased H_2O_2-induced intracellular ROS levels regardless of H_2O_2 concentrations (Figure 2B and 2C).

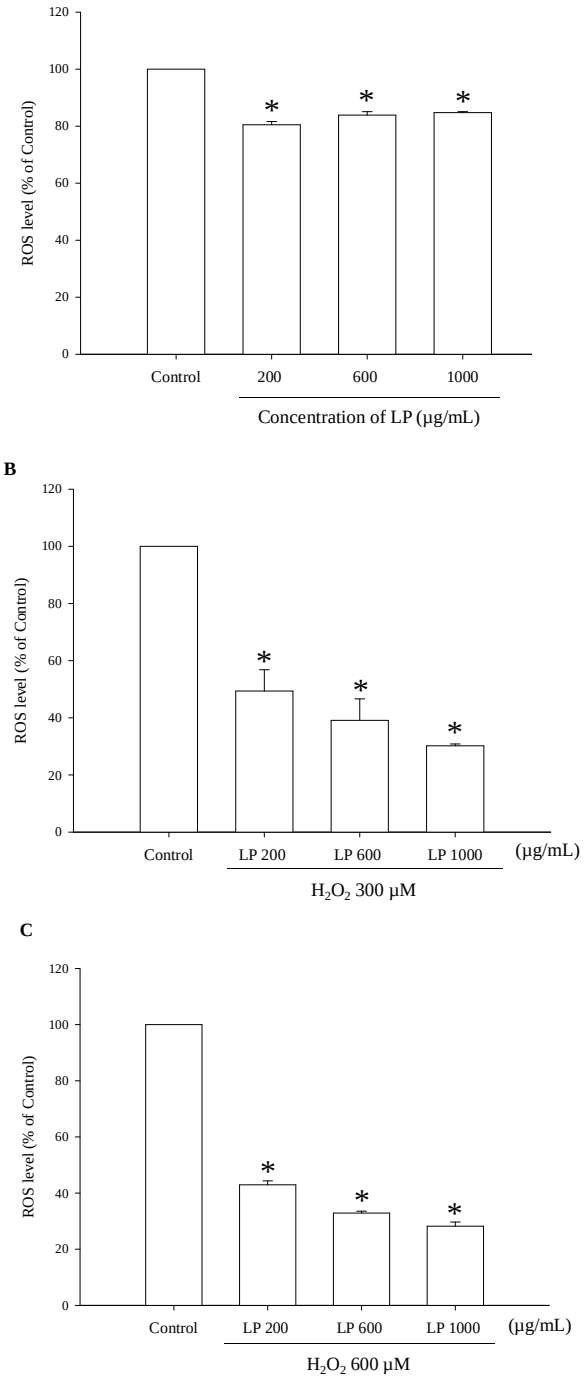

Figure 2: Effects of LP on ROS level in SK-N-SH cells. Cells were preincubated with LP at 200, 600 and 1,000 µg/mL, followed by 30 min incubation with 0 µM (A**)**, 300 µM (B) or 600 µM H_2O_2 (C). Data are presented as mean ± S.E.M. **P* < 0.05 compared to the control

Effect of LP on fear memory: Passive avoidance test

The effects of LP on fear memory are shown in Figure 3. Mice, orally fed with LP 90 or 180 mg/kg/day for 15 days, showed a significant increase in percentage change of latency time on the test day when compared to the control (LP0) group, indicating that LP extract could increase learning and memory ability of mice. Animals injected with 2 mg/kg/day of scopolamine for 15 days showed a significant decrease in percentage change of latency time on test day when compared with the control group. Interestingly, mice that received concomitant 180 mg/kg LP and scopolamine, showed a significant increase in percentage change of latency time when compared with those treated with scopolamine alone, indicating that 180 mg/kg LP, but not 90 mg/kg LP, could attenuate scopolamine-induced fear memory impairment.

Morris water maze test

The effects of LP extract on escape latency of trial 1 are shown in Fig 4. Mice treated with LP at 180 mg/kg/day showed a significant increase in learning and memory ability as seen by a significant decrease in escape latency time when compared to the control group (0 mg/kg LP) on day 16. On days 17 and 18, every group of animals except the scopolamine-treated group, showed a decreased escape latency time when compared to the same treatment on day 16. Interestingly, LP extract at doses of 90 and 180 mg/kg improved chronic scopolamine treatment-induced learning and memory impairment. The results of trials 2 and 3 were in the same pattern as trial 1 (data not shown).

DISCUSSION

This study demonstrated that the aqueous extract of Luem Pua rice (LP) had no effect on viability of SK-N-SH cells. It exhibited significant ROS scavenging properties and provided protection against H_2O_2-induced intracellular ROS in SK-N-SH cells. In the *in vivo* models, LP produced significant improvement of learning and memory in both passive avoidance and Morris water maze tests. LP also antagonized chronic scopolamine-induced memory deficits.

Scopolamine, a traditional muscarinic receptor antagonist, is widely used as a primary screening test for anti-amnesic drugs [22]. Recently, some studies have demonstrated that memory impairment in the scopolamine-induced animal model is associated with increased oxidative stress within the brain [3,23-25]. In addition, scopolamine treatment also increased biological markers of AD including amyloid beta and tau proteins [26]. As shown in the cell culture

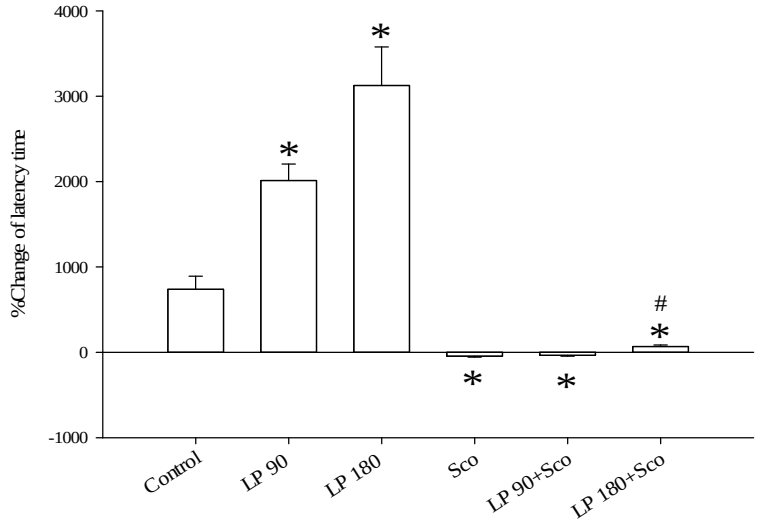

Figure 3: Effects of LP and scopolamine chronic treatment on latency time on the test day in the passive avoidance test are shown. Data are presented as mean ± SEM. * significantly different when compared to the control group; # significantly different when compared to the scopolamine-treated group

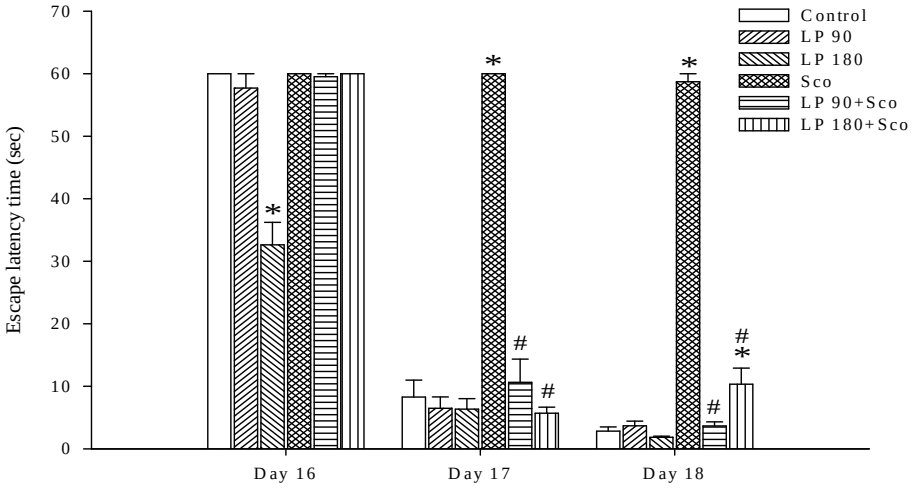

Figure 4: Effects of LP and scopolamine chronic treatment on escape latency time of trial 1, days16-18 of treatment, in the Morris water maze test. Data are represented as percentage change and presented as mean ± SEM. * significantly different when compared to the control group; # significantly different when compared to scopolamine-treated group

experiment, LP extract reversed the effects of scopolamine on memory by reducing the oxidative stress in the brain.

Over the past decades, considerable attention has been focused on the potential of a group of dietary-derived phytochemicals known as flavonoids in modulating neuronal function, thereby increasing memory retention, learning and cognitive functions [27]. In addition, plant flavonoids may delay the development of Alzheimer's disease-like pathology and are suggested to be potential strategies in dementia treatment [28]. Anthocyanins, one of the flavonoid groups predominant in many colored plants, have been credited with capacity to modulate cognitive and motor function, to enhance memory, to prevent age-related declines in neural function [29] and to protect neurons [30]. Luem Pua glutinous rice contains very high anthocyanins, especially in the form of cyanidin-3-glucoside, and high antioxidant activity [19,21]. Although the gastrointestinal absorption of anthocyanins is quite low, they can cross the blood-brain barrier and support antioxidant capacity of the brain and have potential to provide neuro-protection in neuro-degenerative conditions [31]. It might be the case

that the antioxidant activity of the extract was contributing to the memory enhancing effect observed in this study.

CONCLUSION

LP in cell culture shows a significant ROS reducing effect against H_2O_2. The results of the behavior study also suggest that LP has a cognitive enhancing effect. LP also antagonized the deleterious effects of scopolamine on memory. The memory enhancing effect of LP might be through the antioxidant and many other effects of the anthocyanins. This suggests that Luem Pua rice might be developed as a nutraceutical for improving mood and brain function, especially learning and memory.

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