Pig Skin Gelatin Hydrolysates Attenuate Acetylcholine Esterase Activity and Scopolamine-induced Impairment of Memory and Learning Ability of Mice

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Abstract

The protective effect of pig skin gelatin water extracts (PSW) and the low molecular weight hydrolysates of PSW generated via enzymatic hydrolysis with Flavourzyme® 1000L (LPSW) against scopolamine-induced impairment of cognitive function in mice was determined. Seventy male ICR mice weighing 20–25 g were randomly assigned to seven groups: CON, control; SCO, scopolamine (1 mg/kg body weight (B.W.), intraperitoneal (i.p.)); THA 10, tacrin (10 mg/kg B.W., per oral (p.o.)); PSW 10 (10 mg/kg B.W., p.o.); PSW 40, (40 mg/kg B.W., p.o.); LPSW 100 (100 mg/kg B.W., p.o.), and LPSW 400, (400 mg/kg B.W., p.o.). All treatment groups, except CON, received scopolamine on the day of the experiment. The oxygen radical absorbance capacity of LPSW 400 at 1 mg/mL was 154.14 μM Trolox equivalent. Administration of PSW and LPSW for 15 weeks did not significantly effect on physical performance of mice. LPSW at 400 mg/kg significantly increased spontaneous alternation, reaching the level observed for THA (10 mg/kg B.W.) and CON. The latency time of animals receiving LPSW 400 was higher than that of mice treated with SCO alone in the passive avoidance test, whereas it was shorter in the water maze test. LPSW 400 increased acetylcholine content and decreased acetylcholine esterase activity ($p < 0.05$). LPSW 100 and

LPSW 400 reduced monoamine oxidase-B activity. These results indicated that LPSW at 400 mg/kg B.W. is a potentially strong antioxidant and contains novel components for the functional food industry.

Keywords: hydrolysates, pig skin gelatin, cognitive, scopolamine, learning and memory

Introduction

Memory is a cognitive process that can be studied throughout an individual's life span, and cognitive skills are continually used to adapt to an ever changing environment (Sharma, 2009). With age, reduction in cognitive function is one of the changes from loss of neuronal function. Cholinergic neurons in the brain are associated with learning and memory, executive functioning, behavior, and emotional responses. Acetylcholine (ACh) is the principal neurotransmitter in the peripheral, central, somatic, and autonomic nervous systems. Loss of ACh results in cognitive dysfunction such as dementia or Alzheimer's disease (AD) (Bierer et al., 1995). Acetylcholine esterase (AChE) catalyzes ACh, and many scientists have identified functional compounds, such as peptides/hydrolysates, flavonoids, and vitamin E, to inhibit AChE (Ali Reza et al., 2018; Chen et al., 2015; Pervin et al., 2014; Kim et al., 2013; Srividhya et al., 2012; Pei et al., 2010). The brain is especially vulnerable to oxidative stress because of its high oxygen utilization (Feng and Wang, 2012). Moreover, oxidative stress induces biological cell damages, such as oxidation of protein, lipid, DNA and glycooxidation, which

55 are associated with AD (Ali Reza et al., 2018). Dietary antioxidants may play an important role
56 in retarding several cognitive disorders associated with neuronal diseases, including dementia
57 according to the experimental and clinical data (Meydani, 2001). For example, the neuronal
58 and cognitive dysfunctions associated with aging have been shown to be retarded in animals
59 fed dietary supplements of vitamin E or extracts of fruits and vegetables (Joseph et al., 1999),
60 defatted walnut meal hydrolysates (Chen et al., 2015), egg white protein hydrolysates
61 (Martinez et al., 2019), fish peptides (Pei et al., 2010) with high antioxidant activity and
62 cholinergic system maintenance properties (Floyd and Carney, 1992). Thus, natural dietary
63 products might retard the AD by concomitantly protecting brain cells from oxidative stress and
64 acting as cholinesterase inhibitors (Costa et al., 2013; Pervin et al., 2014)

65 Collagen has attracted considerable attention as a bio-material for drug delivery and
66 tissue engineering due to its low antigenicity (Li et al., 2004). Collagen can be transformed into
67 gelatin, which is consumed as a food source, via heat treatment. Gelatin hydrolysates generated
68 using edible enzymes are natural additives and approved by the Food and Drug Administration
69 (FDA) (Dybka and Walczak, 2009). In fact, gelatin hydrolysates containing soluble peptides
70 are manufactured using proteolytic enzymes via controlled hydrolysis. These hydrolysates have
71 been considered to possess beneficial biological properties such as antioxidant (Chang et al.,
72 2013) and bone growth enhancing abilities (Leem et al., 2013). The peptides in gelatin
73 hydrolysates are readily absorbed by the blood from the gastrointestinal tract, thereby

74 becoming available for metabolic processes, whereas gelatin is difficult to absorb (Zague,
75 2008). Previously, we have shown that pork skin gelatin hydrolysates generated using
76 enzymatic hydrolysis with Flavourzyme®1000L possess antioxidant activity (Kim et al., 2013).
77 Moreover, antioxidants have a significant potential of reducing the symptoms and incidence of
78 AD (Ali Reza et al., 2018; Pervin et al., 2014), According to the study by Ali Reza (2018),
79 potential antioxidants from plant sources showed a high correlation between AChE inhibition
80 activity (r^2=0.978) and DPPH radical scavenging activity (r^2=0.998). However, studies on the
81 supplementary effect of water-extracted gelatin hydrolysates on learning and memory function
82 of mice are limited.

83 Therefore, the aim of this study was to elucidate the antioxidant activity and protective
84 effect of water-extracted pig skin gelatin hydrolysates (low molecular weight) generated by
85 food enzymes against scopolamine-induced damage of memory and cognitive function in mice.
86

87 ## Materials and Methods

88 **Production of pig skin gelatin water extracts and low molecular weight pig skin gelatin**
89 **water extract**

90 Pig skin gelatin water extracts (PSW) and low molecular weight pig skin gelatin water
91 extract (LPSW) were prepared by the procedure of Kim et al. (2013) with some modifications.
92 Fat-trimmed pig dorsal skin was extracted thrice in hot water at 100°C for a total of 7 h- 2 h

(first extraction), 2 h (second extraction), and 3 h (third extraction). After every extraction, fresh water was added and all the extracts were mixed. The fat in the extracts was discarded using Folch's solution (chloroform: methanol, 2:1(v/v)). Then, the extract was lyophilized and used as PSW for analysis. For generating LPSW, the lyophilized PSW was swollen in eight-fold excess distilled water at 80°C and stirred for 1 h and adjusted to pH 7.0. Flavorzyme®1000L (0.3% (w/w); Novozymes, Bagsvaerd, Denmark) from *Aspergillus oryzae* was subsequently added and the mixture was incubated for 12 h at 50°C. Enzymatic hydrolysis was stopped by heating at 95°C for 10 min. The hydrolysates centrifuged at 4,000 × *g* for 30 min using Amicon® Ultra-15 centrifugal filter units (Merck Millipore, Bedford, MA, USA) and the 3 kDa molecular weight filtrate was lyophilized and used as LPSW.

Oxygen radical absorbance capacity of PSW and LPSW

The oxygen radical absorbance capacity (ORAC) assay kit (Cell Biolabs, Inc., San Diego, CA, USA) was used to determine ORAC value according to manufacturer's instructions. Results were presented as μM Trolox equivalent (TE)/g sample.

Animals and experimental design

Seventy male ICR mice weighing 20–25 g were randomly assigned to seven groups (Fig. 1): CON, control; SCO, scopolamine (1 mg/kg body weight (B.W.), intraperitoneally

(i.p.)); THA 10, tacrin (10 mg/kg B.W., per oral (p.o.)); PSW 10 (10 mg/kg B.W.); PSW 40, (40 mg/kg B.W.); LPSW 100 (100 mg/kg B.W.); LPSW 400 (400 mg/kg B.W.). Each group consisted of ten mice, which were housed in wire cages and maintained on a 12 h day/night cycle with free access to food and water at constant temperature (23°C ± 1) and humidity (50–60%) for 15 weeks. All measurements were made between 10:00 and 18:00 h. Scopolamine hydrobromide (Sigma-Aldrich, UK), a well-known muscarinic receptor blocker that impair learning and memory functions in both animals and human beings, was dissolved in 0.9% sterilized saline at a dose of 1.0 mg/kg. The dissolved scopolamine (0.2 mL) was then injected intraperitoneally 30 min prior to the experiment. Tacrine, the drug for cure of AD, was used as a positive control to compare the enhancing effect of PSW and LPSW. All animal experiments were performed under Kangwon National University's Committee on the Care and Use of Laboratory Animals Guidelines (KIACUC-12-0011) and analytical grade of chemicals and reagents were used.

Memory enhancing behavior test

Immediate spatial working memory (Y-maze test)

The Y-maze test is used to determine short-term memory (immediate spatial working memory) (Rao et al., 2005). Spatial memory contributes to an animal's knowledge and exploration of the available resources in its surroundings (Sharma, 2009). The Y-maze comprises of a three-arm

horizontal maze (40 cm long and 3 cm wide with 15 cm-high walls) in which the arms, labeled A, B, and C, are symmetrically disposed at 120° to each other. The number and sequence of arm entries made during each 8-min session were recorded. Alternations were regarded as an entry into each arm within three consecutive arm choices such as A-B-C or B-C-A. Percentage of alternation was calculated as the number of alternations divided by the number of total arm entries minus two, as calculated using the equation as follows. The number of arm entries was considered as the indicator of locomotor activity.

Alternation = [(Number of alternations)/ (Total arm entries-2)] × 100.

Passive avoidance test

The passive avoidance test was performed by the method of Das et al. (2005). This test was conducted by a shuttle box [(410 (w) × 201 (D) × 300 mm (H)] comprised of two compartments: an illuminated place with a 60 W bulb and a dark compartment consisting of 2-mm stainless steel rods with 1 cm apart. A guillotine door was used to isolate the compartments. One hour after the last administration of the test materials, for the acquisition trial, each mouse was in turn gently placed in the illuminated place and the door was opened after 10 s. When mice entered the dark place, the door was manually closed and an electrical shock (0.5 mA) of 3 s duration was delivered through the stainless steel rods. The time taken for the mouse in the dark place was recorded as the initial latency time. Twenty-four hours after this acquisition trial,

the mouse was again placed in the illuminated place for a retention trial. The time taken for the mouse to enter the dark place after the door was opened was regarded as the retention latency time for both trials. The retention latency time to enter the dark place was listed up to 180 s. If a mouse did not enter the dark place within 180 s, it was regarded as a retention latency time score of 180 s.

Morris water maze test

The Morris water maze is a useful behavioral test for assessing spatial learning ability associated with septohippocampal cholinergic activity (Li et al., 2001). It was conducted in a pool of 107-cm diameter with a circular acrylic platform (10 cm in diameter) submerged 1 cm below the surface of the opaque water at 23 ± 2°C. Mice were allowed two acquisition trials per day for four days. Movement of mice in the water maze was captured using a camera and evaluated manually using a clock timer during each trial. The mice were allowed to stay for 10 s on to the platform, when they found hidden platform beneath the opaque water. When the mice failed to find the platform within 120 s, they were placed on the platform by the experimenter for a maximum of 30 s. A day after the last training trial session, the mice were subjected to the pool in which the platform was removed and the animals were allowed to swim for 120 s searching for it. The swimming time in the pool quadrant where the platform had previously been placed was kept recoding.

Blood profile and organ weight of mice

After completion of the behavior test, mice were anesthetized using diethyl ether and blood was taken by heart puncture. The blood was centrifuged at 890 × *g* for 15 min, and the serum total protein, glutamic oxaloacetic transaminase (GOT), and glutamic pyruvic transaminase (GPT) levels were determined using the ADVIA 2400 chemistry system (Siemens, USA). The brain, liver, lungs, kidneys, spleen, and testes were dissected, weighed, and expressed as relative organ weight (with respect to body weight).

Cerebral substrate concentration and enzyme activities

Preparation of brain tissue homogenate

Brain homogenates were prepared using the method of Kim et al. (2010). Whole brain tissue (n = 5) was homogenized in 12.5 mM sodium phosphate (pH 7.0) buffer containing 400 mM NaCl using a Teflon tissue grinder at 4°C. The whole brain homogenate was used for determination of the ACh content, AChE activity, and monoamine oxidase-B (MAO-B) activity.

ACh content and AChE activity

Homogenate of the brain tissue was centrifuged at 10,000 × *g* for 20 min at 4°C. The supernatant was used to determine ACh content using the EnzyChrom ACh assay kit (EACL-

100, Bioassay System, CA, USA).

AChE activity was determined using the method of Ellman et al. (1961) with slight modifications. The brain tissue homogenate was centrifuged at 1,000 × *g* for 10 min at 4°C. For the reaction, 260 µL of 100 mM sodium phosphate buffer (pH 8.0), 20 µL of 10 mM 5-5'-thiobis-2-nitrobenzoic acid (DTNB), 10 µL of brain tissue supernatant, and 10 µL of 100 mM ACh chloride were added. ACh (10 µL, 100 mM) was added before starting the reaction and the absorbance was subsequently detected at 412 nm using a UV/visible microplate reader (Spectra Max M2e, Molecular Devices, Sunnyvale, CA, USA). The reading was repeated at 15 s intervals to verify that the reaction occurred lineally.

Monoamine oxidase-B (MAO-B) activity

After centrifugation (10,000 × *g* for 20 min at 4°C) of the brain tissue homogenate, the pellet was used to assay MAO-B activity in brain tissue using the Amplex Red monoamine oxidase assay kit (A-12214, Molecular Probes, OR, USA). In brief, a reaction mixture (500 µL) containing Amplex Red reagent (400 µM), benzylamine (2 mM) as a specific substrate for MAO-B, and horseradish peroxidase (2 U/mL) was prepared. The mixtures were incubated at 23°C in 96-well plates for 1 h. Fluorescence was then measured using a fluorescence reader (Molecular Device, USA) at excitation wavelength of 560 nm ± 10 nm and emission wavelength of 590 ± 10 nm. The MAO-B activity was recorded as µM resorufin/60 min/mg

207 protein. The protein content of the sample was assayed using the bicinchoninic acid (BCA)
208 protein assay kit (Sigma, MA, USA).
209
210 **Statistical analysis**
211 All data were analyzed using the SAS software with a general linear model procedure
212 (GLM). The mean values and standard errors were reported. To compare the mean values,
213 Duncan's multiple range test was used and $p < 0.05$ was considered statistically significant.
214
215 ## Results and Discussion
216 **Yield and ORAC value of pig skin hydrolysates (PSW and LPSW)**
217 The yields and protein content of PSW and LPSW were 27.3 and 11.6%, and 525.7±10.73 and
218 58.9±0.51 mg/g dry mass, respectively (data not shown). The ORAC values of PSW and LPSW
219 increased in a dose dependent manner (Fig. 2). The ORAC value of PSW and LPSW at 1
220 mg/mL was 40.14 μM TE/g sample and 154.14 μM TE/g sample, respectively. The low
221 molecular weight hydrolysates of < 3 kDa showed significantly higher ORAC value. This is in
222 accordance with the results of Kim et al. (2013), who reported that the ORAC values of 1
223 mg/mL pig skin gelatin hydrolysates (more than 50 kDa) and low molecular weight pig skin
224 gelatin hydrolysates (less than 3 kDa) were 29.31 μM TE/g sample and 141.39 μM TE/g sample,
225 respectively. Joseph et al. (1998) reported that supplementation of AIN93 diet with spinach

226 extracts containing high antioxidant activity, assessed by the ORAC assay, prevented the onset
227 of age related deficits in several indices (signal transduction) and cognitive behavior (Morris
228 water maze performance).
229
230 **Body weight gain, feed intake, and feed efficiency ratio of mice**
231 Body weight gain, feed intake, and feed efficiency of mice are shown in Table 1. The
232 results suggested that PSW and LPSW supplementation did not affect the physical condition
233 of mice during the experiments.
234
235 **Mice behavior test for memory enhancing activity**
236 **Y-maze test**
237 Y-maze spontaneous alternation is a behavioral test for determining the willingness of
238 rodents to explore new environments (Ru and Liu, 2018). Mice prefer to investigate a new arm
239 of the maze rather than returning to an arm that has been visited previously. Many parts of the
240 brain, including the hippocampus, septum, basal forebrain, and prefrontal cortex, are involved
241 in this task (Sharma, 2009). The number of total entries (A) and the spontaneous alternation
242 ratio (B) of mice fed PSW and LPSW are shown in Fig. 3. The spontaneous alternation ratio
243 of mice in the LPSW 400 group was significantly higher than that of the SCO group and was
244 similar to the ratio of the CON and THA groups ($p < 0.05$). This suggests that LPSW 400

245 attenuated the decline in scopolamine-induced spatial working memory of mice. In our
246 previous study, administration of 2% gelatin hydrolysates (molecular weight 3–50 kDa)
247 isolated from pig skin gelatin after 12 h hydrolysis using a 1:1 mixture of Alcalase and
248 Protamex significantly increased alternation behavior by up to 13.5% (Jang et al., 2011).
249
250 **Passive avoidance test**
251 The latency time of mice supplemented with PSW and LPSW in terms of passive
252 avoidance test training and test trial is shown in Fig. 4. During the training, no significant
253 difference was observed; however, the latency time required to resist the black compartment
254 was significantly higher in the LPSW 400 group than in the SCO, PSW 40, and LPSW 100
255 groups. This demonstrated that dietary administration of LPSW 400 may facilitate the
256 acquisition of spatial leaning and increase passive avoidance ability, increasing these to the
257 level observed for THA, although these values did not reach up to the level of the CON group.
258 Pei et al. (2010) reported that supplementation with 0.22, 0.44, and 1.32% (w/w) marine
259 collagen peptide (MCP) for 3 months significantly enhanced the learning ability of aged mice.
260 They also reported that the MCP significantly alleviated oxidative stress, reduced the number
261 of apoptotic neurons, and up-regulated the expression of brain-derived neurotrophic factor.
262
263 **Morris water maze test**

The water maze task is a common and sensitive behavioral test used for assessing the spatial learning and memory of experimental animals (Li et al., 2001). To complete the task, animals must locate a hidden platform during a series of trials. Once animals learn where the hidden platform is located, they can remember this location and swim rapidly to it from any starting point. The time taken to reach the platform was measured. As shown in Fig. 5A, the mean latency time to find the platform decreased progressively during the four training days in all animals. Mice in the control group showed a significant decrease in latency time during the acquisition trial ($p < 0.05$). On the fourth day after training for 3 days, mice in the SCO group showed significantly longer latency time (79.83 s); however, mice in both the LPSW 100 (53.17 s) and LPSW 400 (24.17 s) groups showed significantly shorter latency time than those in the SCO group. The latency time of the LPSW 400 group was significantly shorter than that of the LPSW 100 group, which were similar to those of CON (13.83 s) or THA (31.37 s) groups, respectively. This suggested that LPSW 400 significantly enhanced Morris water maze performance against learning and memory impairment induced by scopolamine, and was as effective as THA. On the day of the test trial, mice swam in the vicinity of the place where the platform had been located during the acquisition trial. As shown in Fig. 5B, mice in the LPSW 400 group showed higher search precision for the platform than mice in the SCO, PSW 40, and LPSW 100 groups did ($p < 0.05$), which was comparable to those of mice of the CON and THA groups. Therefore, supplementation of LPSW at 400 mg/kg appeared to restore the

283 scopolamine-induced loss of spatial memory in mice.

284

285 **Blood profile and relative organ weight**

286 Scopolamine is used as an amnesia inducer due to its adverse effects on cognitive
287 function such as vomiting, nausea, weight loss, and hepatotoxicity (Mendiola-Precoma et al.,
288 2016). Tacrine, used as a positive control in this study, was the first drug approved for the
289 treatment of AD since 1993. However, it was withdrawn in 2013 because of its hepatotoxicity
290 (de los Ríos and Marco-Contelles, 2019). To assess hepatotoxicity in mice injected with tacrine
291 and scopolamine, and that after supplementation with PSW and LPSW, the serum total protein,
292 and GOT and GPT levels of mice were estimated (Table 2). The serum total protein content of
293 mice in the CON and SCO groups did not differ. However, mice in the THA 10 group showed
294 significantly higher total protein content due to hepatotoxicity. Neither concentration of PSW
295 and LPSW induced any significant difference in serum protein content. In addition, LPSW 400
296 treatment did not significantly alter the serum protein contents of the CON and SCO groups,
297 indicating that administration of LPSW 400 mg/kg did not change serum protein content. The
298 serum GOT level of mice dosed with THA and PSW 10 were significantly higher than those of
299 control animals and those receiving LPSW400. Furthermore, LPSW 100 and 400 significantly
300 reduced the GOT level up to that observed in the control group, whereas no significant
301 difference was observed when compared to the level in the SCO group. Serum GPT level was

not affected by scopolamine (i.p.), tacrine (i.p.), PSW (p.o.), and LPSW (p.o.) treatments.

The relative weights of the liver, kidneys, spleen, lungs, testes, and brain of the test animals are shown in Table 3. There was no significant difference in relative weight among the treatment groups. Organ weight can be the most sensitive indicator of the effect of an experimental compound, as significant differences in organ weight between treated and untreated (control) animals may occur in the absence of any morphological changes (Bailey et al., 2004). Our results indicated that supplementation of PSW and LPSW at 100 and 400 mg/mL concentration did not change the organ weight in mice.

Brain substrates and enzymes

Cerebral ACh contents

The cerebral ACh content in the brains of mice dosed with LPSW 400, THA, and CON were significantly higher than those dosed with SCO (Table 4). However, no significant difference was observed among CON, THA 10, PSW 40, LPSW 100, and LPSW 400. Several recent studies have considered the effect of dietary supplementation on the cholinergic system during aging (Willis et al., 2009). ACh is synthesized in pre-synaptic terminals from choline and is required for cholinergic neurotransmission in the central and peripheral nervous systems (Goodman & Soliman, 1991; Srividhya et al., 2012). Shortage of ACh in the brain has been associated with AD. The cholinergic system is strictly dependent on both oxidative metabolism

and choline supply (Tucek, 1985; Pervin et al., 2014).

Cerebral AChE activity

The cerebral AChE activity in the brains of mice fed PSW and LPSW is shown in Table 4. THA and LPSW 100 and 400 effectively reduced AChE activity to 25% and 26.7%, respectively, which were equivalent to the activity observed in the CON group (23.4%) compared to the activity of SCO group as 100%. However, no significant effect was observed in the treatment groups. We observed that the ACh content increased when the AChE activity decreased. This observation is in agreement with the fact that AChE is an enzyme that catalyzes ACh, and indicated that LPSW 400 affected the cholinergic system, which is highly dependent on oxidative metabolism and ACh release (Pervin et al., 2014).

The hydrophilic species are the major forms of AChE in the brain, muscle, and other tissues, which forms disulfide-linked oligomers with collagenous or lipid-containing structural subunits (Sussman et al., 1991). AChE plays an important role in the ACh-cycle, including in the release of ACh (Srividhya et al., 2012). Jang et al. (2011) reported that supplementation of 1, 2, and 4% pig skin gelatin hydrolysates (molecular weight between 3 kDa and 50 kDa, obtained via hydrolysis of pig skin gelatin by Alcalase™ and Protamex™) for 16 weeks significantly reduced AChE levels in the ICR mice brain to 48.9, 47.8, and 52.1%, respectively.

MAO-B assay

MAOs are enzymes located in the mitochondria of the liver and other tissues and modulate the level of neurotransmitters such as dopamine, norepinephrine, and serotonin. Hence, many studies have been attempted to inhibit the MAOs, the levels of which increase with age, for the treatment of central nervous system (CNS) disorders (Zhang et al., 2019). MAO-A and MAO-B levels increased by 6-fold in cardiac tissue and by 4-fold in neuronal tissue with age (Zhang et al., 2019). This increased the release of hydrogen peroxide, leading to oxidative stress and degeneration of CNS tissue (Edmondson et al., 2007). We observed that the MAO-B activity in SCO significantly increased to 12.37 μM resorufin/60 min/mg protein, whereas MAO-B activity in THA, LPSW 100, and LPSW 400 significantly decreased and showed activity similar to that of CON (Table 4). These results demonstrated the beneficial role of pig skin gelatin hydrolysates in the formation of the neurotransmitter ACh by decreasing AChE levels as shown in Table 4, leading to significant reduction in MAO-B activity. Zhang et al. (2019) suggested that dietary antioxidative phenolics such as resveratrol and pterostilbene can also reduce MAO-A and MAO-B levels, respectively.

Conclusion

The low molecular weight hydrolysates generated from pig skin gelatin using Flavourzyme® 1000L can be used as dietary compounds for protecting ACh in brains from

AChE, reducing MAO-B activity, and attenuating memory and learning deficit induced by scopolamine. However, this study is preliminary and additional studies are required to understand the metabolic events and gene expression changes occurring after administration of specific peptides from the hydrolysates and their processing by the intestinal and cognitive systems.

Ethics Approval

Animal experiment performed in this study was approved by IACUC (KIACUC-12-0011).

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454

Figure Legends

Fig. 1. Outline of animal experiment.

i.p., intraperitoneal injection

p.o., per oral

Fig 2. Oxygen radical absorbance capacity of pig skin water extracts and low molecular weight pig skin water extracts.

All values are means±SE.

a-j Means with different letters on the bars different significantly at $p<0.05$.

PSW, pig skin water extracts

LPSW, low molecular weight pig skin water extract

Fig. 3. Number of total entries and spontaneous alternations on Y-maze test.

All values are means±SE.

a-c Means with different letters on the bars different significantly at $p<0.05$.

CON, control; SCO, scopolamine (1 mg/kg B.W., i.p.); THA 10, tacrin (10 mg/kg B.W., p.o.); PSW 10, pig skin water extracts (10 mg/kg B.W. p.o.) with SCO; PSW 40, pig skin water extracts (40 mg/kg B.W. p.o.) with SCO; LPSW 100, low molecular weight pig skin water extracts (100 mg/kg B.W. p.o.) with SCO; LPSW 400, low molecular weight pig skin water extracts (400 mg/kg B.W., p.o.) with SCO.

Fig. 4. Latency time for training (A) and test trial (B) in passive avoidance test of mice fed PSW and LPSW.

All values are means±SE.

a-d Means with different letters on the bars different significantly at $p<0.05$.

CON, control; SCO, scopolamine (1 mg/kg B.W., i.p.); THA 10, tacrin (10 mg/kg B.W., p.o.); PSW 10, pig skin water extracts (10 mg/kg B.W. p.o.) with SCO; PSW 40, pig skin water extracts (40 mg/kg B.W. p.o.) with SCO; LPSW 100, low molecular weight pig skin water extracts (100 mg/kg B.W. p.o.) with SCO; LPSW 400, low molecular weight pig skin water extracts (400 mg/kg B.W., p.o.) with SCO.

Fig. 5. Latency time for training (A) and test trial (B) in water maze test of mice fed PSW and LPSW.

All values are means±SE.

a-e Means with different letters on the lines and bars different significantly at $p<0.05$.

CON, control; SCO, scopolamine (1 mg/kg B.W., i.p.); THA 10, tacrin (10 mg/kg B.W., p.o.); PSW 10, pig skin water extracts (10 mg/kg B.W. p.o.) with SCO; PSW 40, pig skin water extracts (40 mg/kg B.W. p.o.) with SCO; LPSW 100, low molecular weight pig skin water extracts (100 mg/kg B.W. p.o.) with SCO; LPSW 400, low molecular weight pig skin water extracts (400 mg/kg B.W., p.o.) with SCO.

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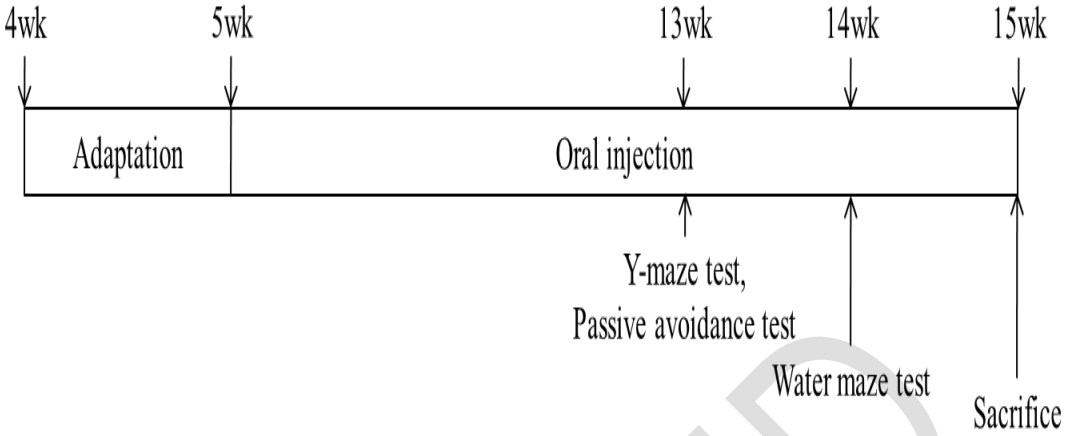


Treatment Groups	
CON	Control
SCO	Scopolamine (1mg/kg, i.p.)
THA10	Tacrine (10mg/kg, p.o.) + SCO (1mg/kg, i.p.)
PSW10	Pig skin gelatin water extracts (10mg/kg, p.o.) + SCO (1mg/kg, i.p.)
PSW40	Pig skin gelatin water extracts (40mg/kg, p.o.) + SCO (1mg/kg, i.p.)
LPSW100	Low molecular weight pig skin gelatin water extracts (100mg/kg, p.o.) + SCO (1mg/kg, i.p.)
LPSW400	Low molecular weight pig skin gelatin water extracts (400mg/kg, p.o.) + SCO (1mg/kg, i.p.)

Fig. 1

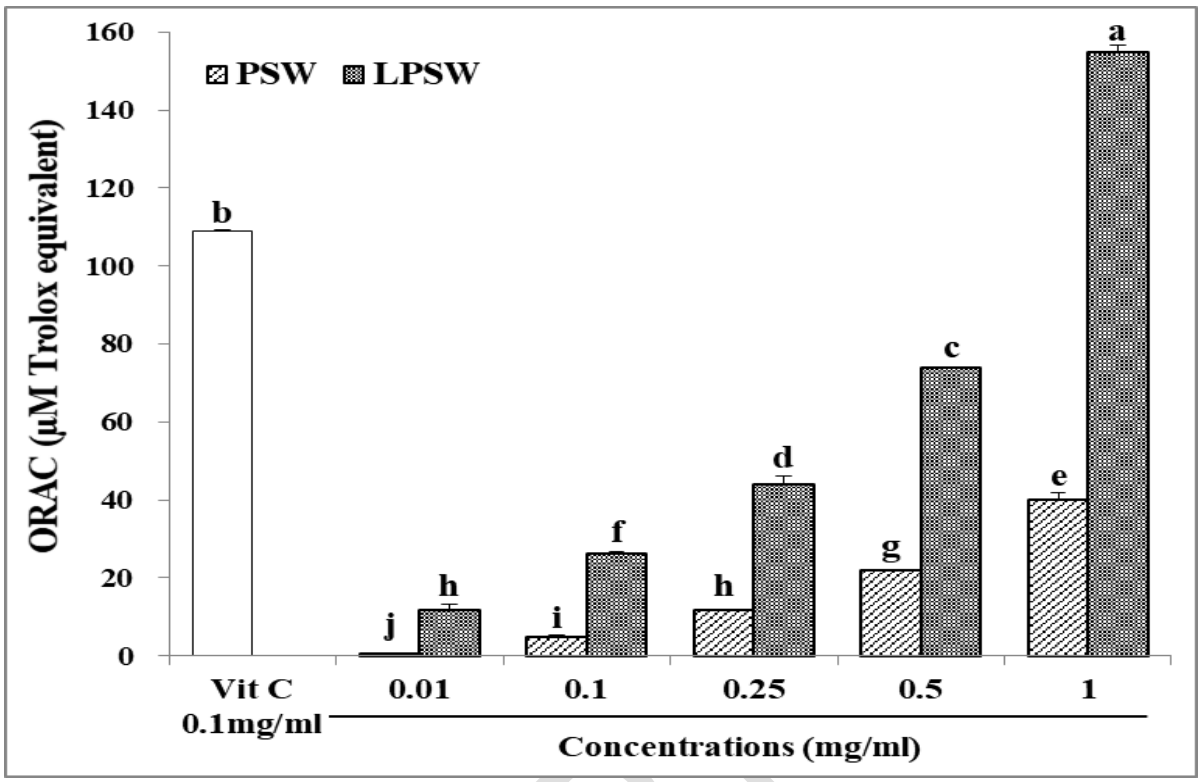
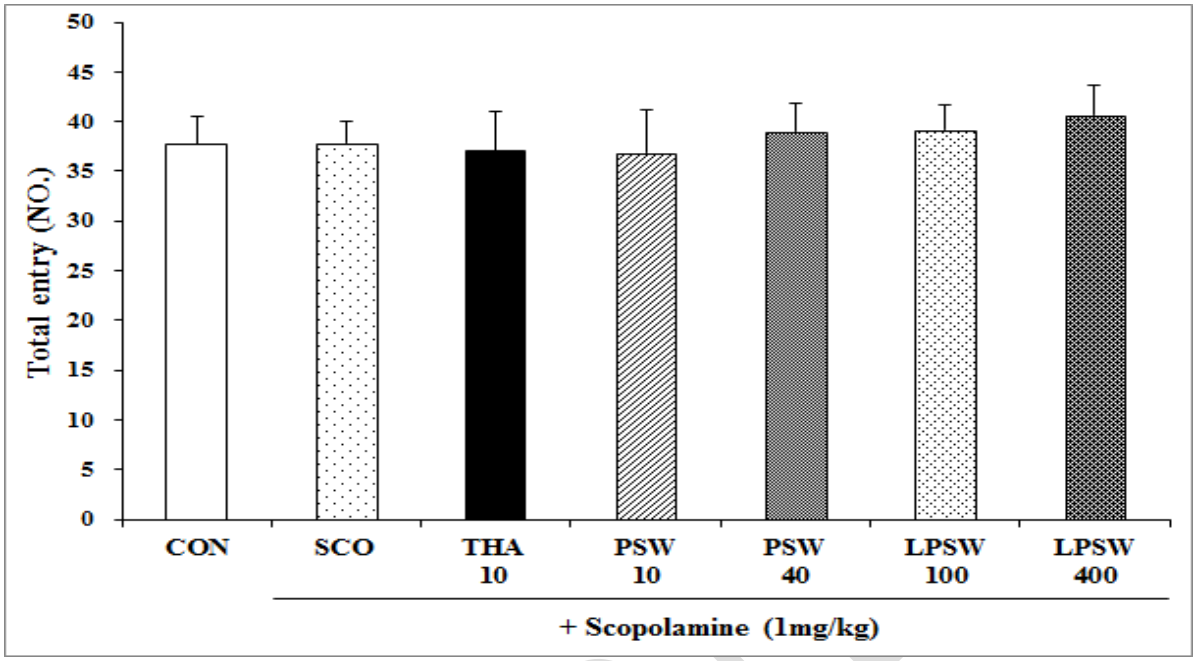

ORAC (µM Trolox equivalent)
160
140
120
100
80
60
40
20
0
PSW
LPSW
b
j
h
i
f
h
d
g
c
e
a
Vit C 0.1mg/ml
0.01
0.1
0.25
0.5
1
Concentrations (mg/ml)

Fig. 2

(A)


Total entry (NO.)
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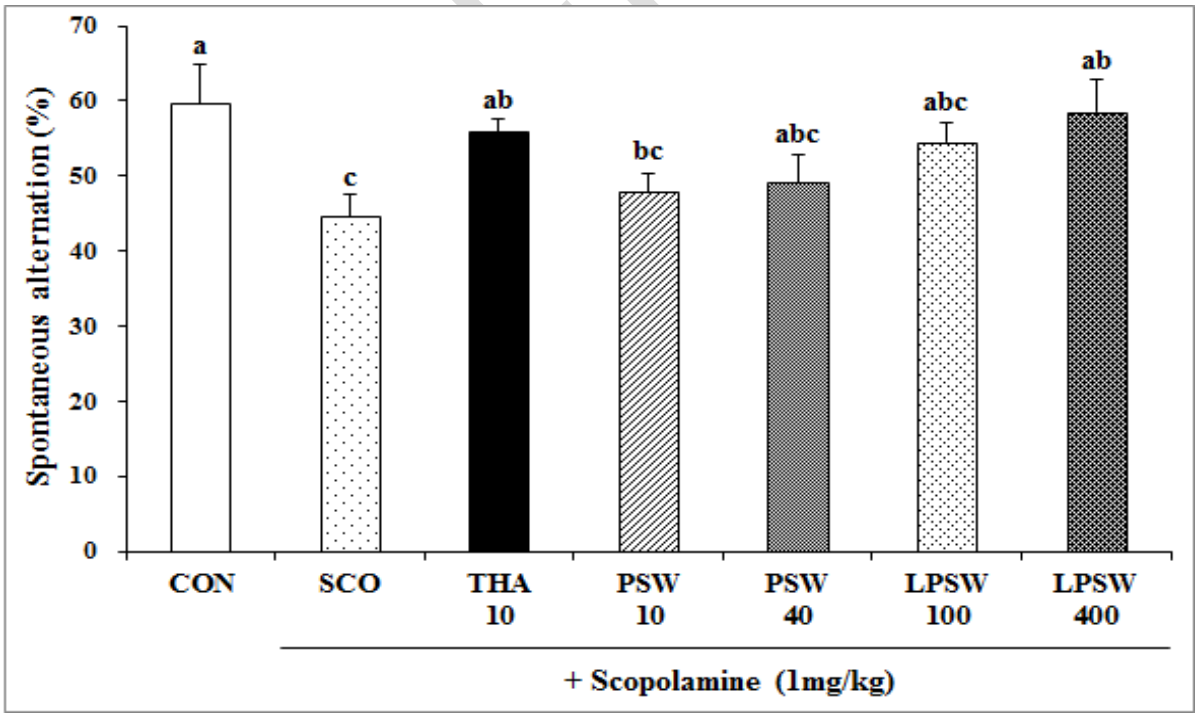
45
40
35
30
25
20
15
10
5
0
CON
SCO
THA 10
PSW 10
PSW 40
LPSW 100
LPSW 400
+ Scopolamine (1mg/kg)

(B)


Spontaneous alternation (%)
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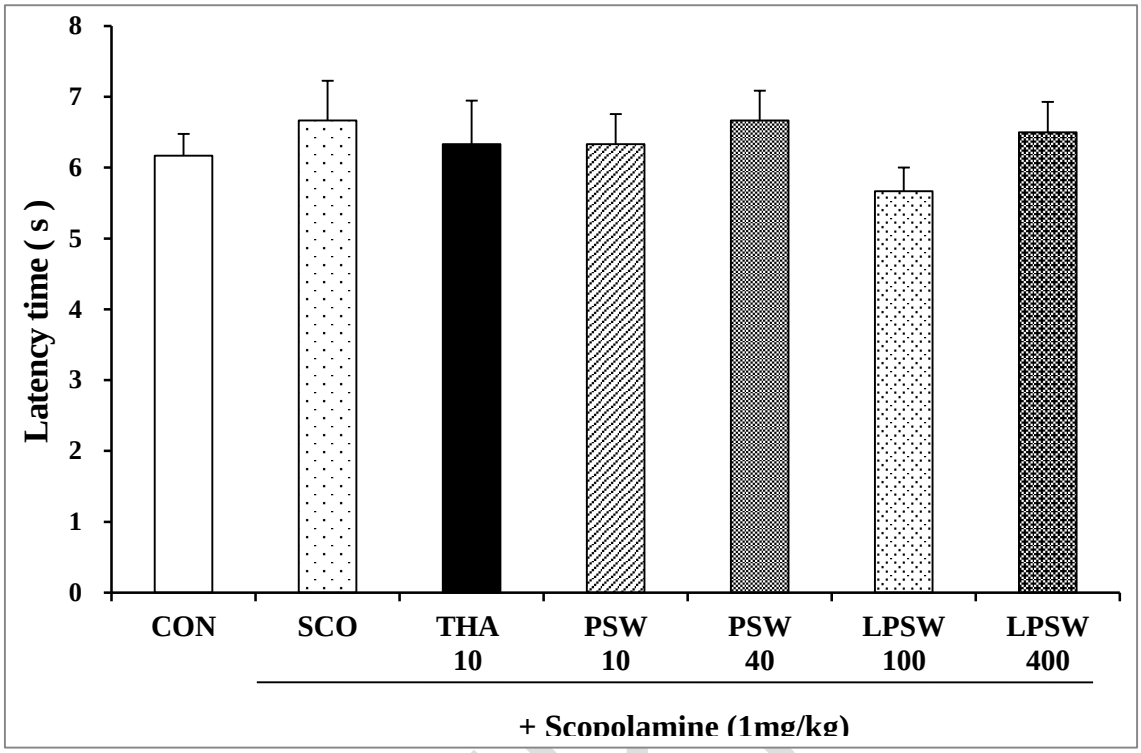
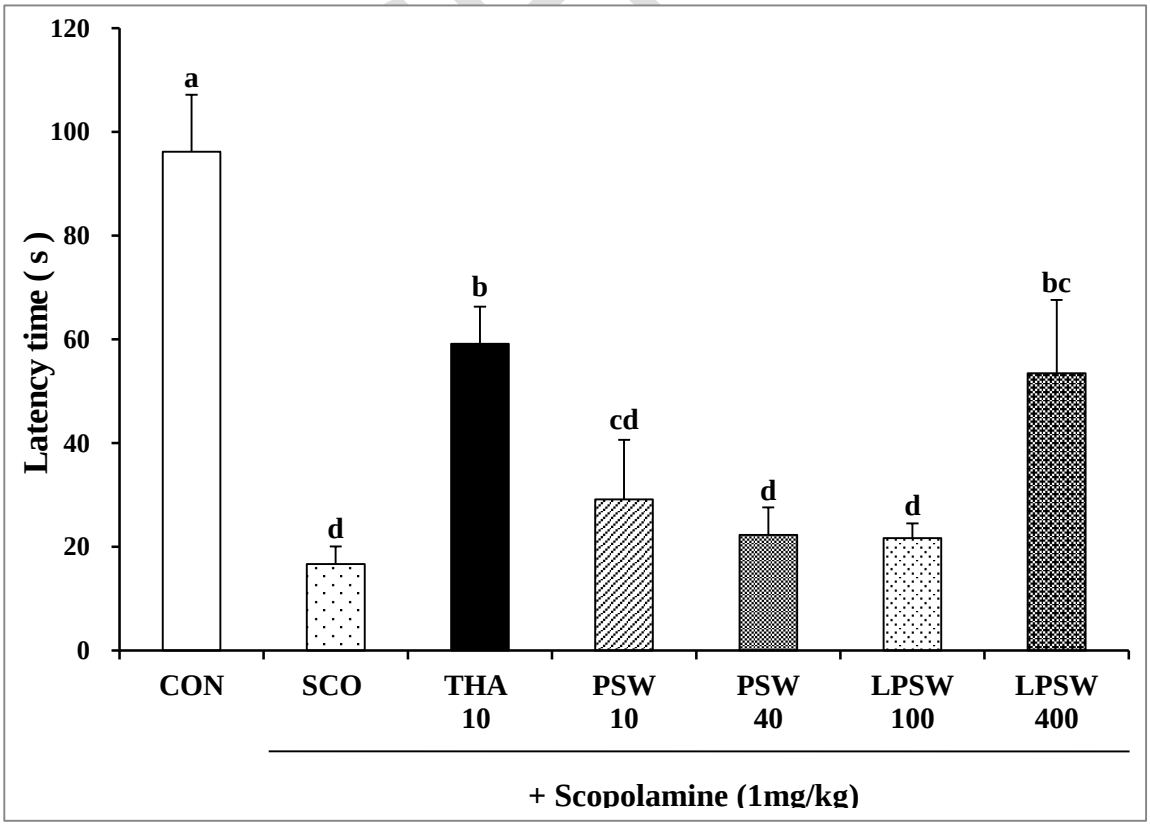
60
50
40
30
20
10
0
a
c
ab
bc
abc
abc
ab
CON
SCO
THA 10
PSW 10
PSW 40
LPSW 100
LPSW 400
+ Scopolamine (1mg/kg)

Fig. 3

(A)


Latency time (s)
8
7
6
5
4
3
2
1
0
CON
SCO
THA 10
PSW 10
PSW 40
LPSW 100
LPSW 400
+ Scopolamine (1mg/kg)

(B)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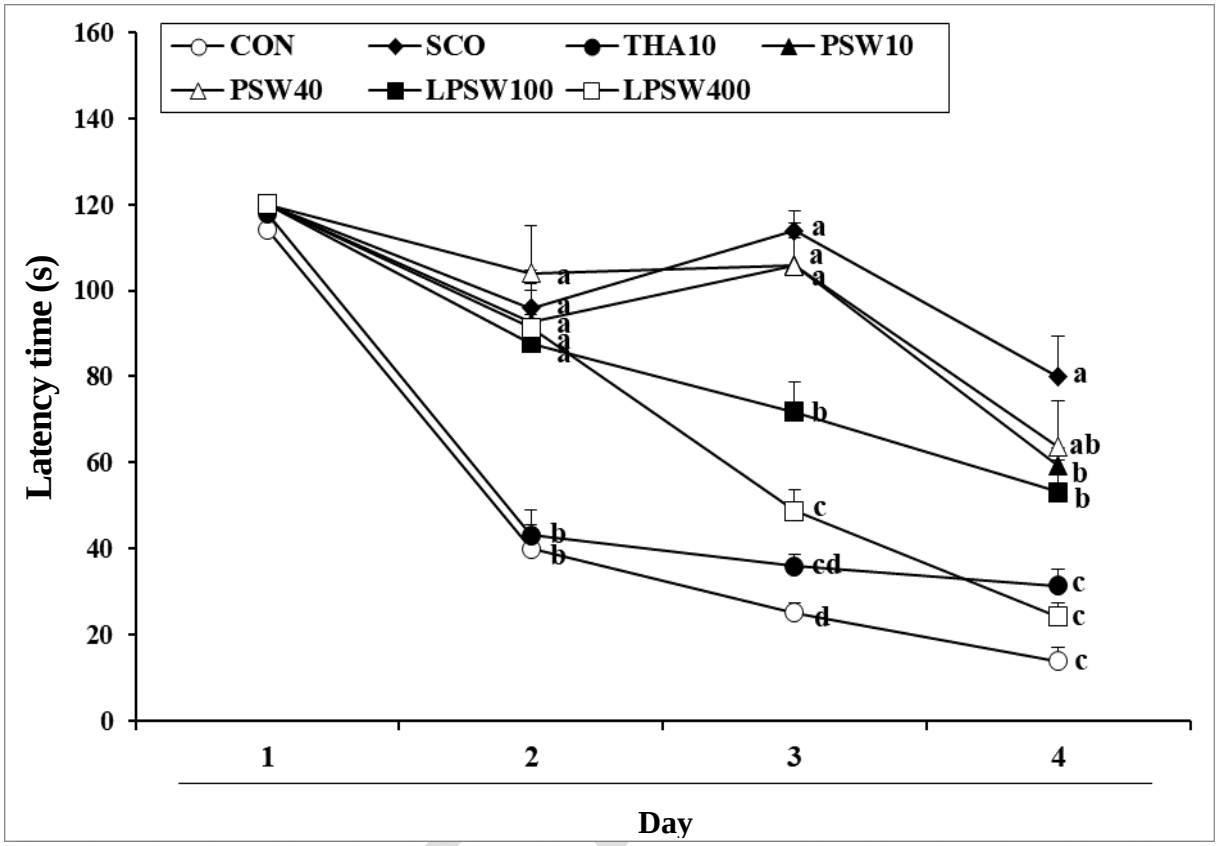
100
80
60
40
20
0
a
d
b
cd
d
d
bc
CON
SCO
THA 10
PSW 10
PSW 40
LPSW 100
LPSW 400
+ Scopolamine (1mg/kg)

Fig. 4

(A)


CON
SCO
THA10
PSW10
PSW40
LPSW100
LPSW400
Latency time (s)
Day
0
20
40
60
80
100
120
140
160
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3
4
a
a
a
a
a
a
a
b
b
b
b
c
cd
c
d
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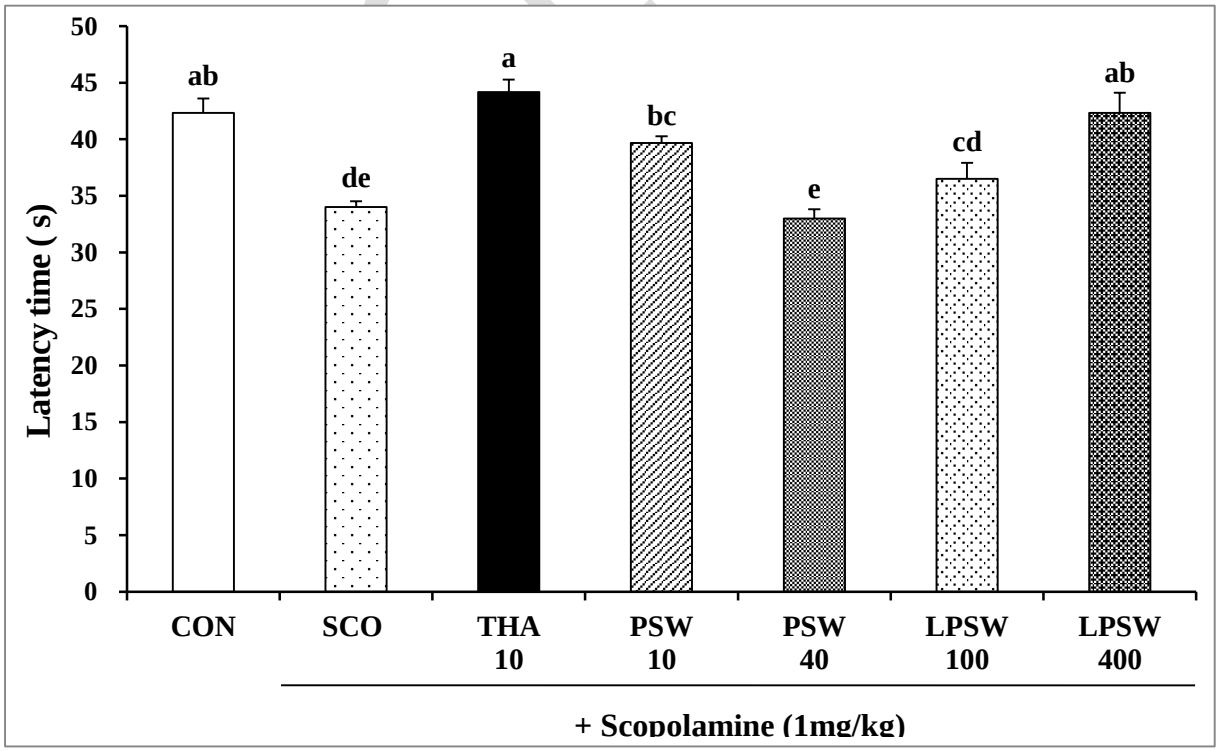
ab
b
c

(B)


Latency time (s)
0
5
10
15
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25
30
35
40
45
50
ab
de
a
bc
e
cd
ab
CON
SCO
THA 10
PSW 10
PSW 40
LPSW 100
LPSW 400
+ Scopolamine (1mg/kg)

Fig. 5

Table 1. Body weight gain, food intake, and food efficiency ratio of of mice fed PSW and LPSW

Treatment[1)]	Body weight gain (g/day)	Food intake (g/day)	FER[2)]
CON	0.14±0.019	5.31±0.070	0.03±0.004
SCO	0.14±0.010	5.36±0.170	0.02±0.002
THA10	0.16±0.019	5.55±0.030	0.03±0.002
PSW10	0.15±0.008	5.62±0.035	0.03±0.002
PSW40	0.16±0.004	5.64±0.110	0.03±0.000
LPSW100	0.15±0.006	5.54±0.080	0.03±0.002
LPSW400	0.14±0.005	5.41±0.220	0.03±0.002

All values are mean ± S.E.

[1)] CON, control; SCO, scopolamine (1 mg/kg B.W., i.p.); THA 10, tacrin (10 mg/kg B.W., p.o.); PSW 10, pig skin water extracts (10 mg/kg B.W. p.o.) with SCO; PSW 40, pig skin water extracts (40 mg/kg B.W. p.o.) with SCO; LPSW 100, low molecular weight pig skin water extracts (100 mg/kg B.W. p.o.) with SCO; LPSW 400, low molecular weight pig skin water extracts (400 mg/kg B.W., p.o.) with SCO.

[2)] FER: feed efficiency ratio (body weight gain/feed intake)

626 **Table 2. Serum total protein content, and GOT and GPT of mice fed PSW and LPSW**

Treatment[1)]	Total protein (g/dL)	GOT (IU/dL)	GPT (IU/dL)
CON	5.97±0.088^{b}	105.33±3.712^{d}	32.67±4.910
SCO	5.97±0.033^{b}	137.00±4.041^{bc}	31.67±2.404
THA10	6.47±0.120^{a}	166.67±0.882^{a}	33.00±1.000
PSW10	6.30±0.153^{ab}	172.33±13.445^{a}	35.67±1.667
PSW40	6.43±0.033^{a}	148.67±16.374^{ab}	31.00±2.082
LPSW100	6.23±0.033^{ab}	130.00±1.528^{bcd}	29.00±2.082
LPSW400	6.13±0.203^{ab}	118.00±7.550^{cd}	30.00±1.528

627 All values are mean ± S.E.

628 $^{a-d}$ Means within same column with different letter differ significantly ($p < 0.05$).

629 [1)] CON, control; SCO, scopolamine (1 mg/kg B.W., i.p.); THA 10, tacrin (10 mg/kg B.W., p.o.); PSW 10, pig skin
630 water extracts (10 mg/kg B.W. p.o.) with SCO; PSW 40, pig skin water extracts (40 mg/kg B.W. p.o.) with SCO;
631 LPSW 100, low molecular weight pig skin water extracts (100 mg/kg B.W. p.o.) with SCO; LPSW 400, low
632 molecular weight pig skin water extracts (400 mg/kg B.W., p.o.) with SCO.
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641 **Table 3. Relative organ weight to body weight of mice fed PSW and LPSW**

Treatment	Liver	Kidneys	Spleen	Lungs	Testes	Brain
CON	3.57±0.054	1.33±0.047	0.26±0.014	0.52±0.028	0.58±0.021	0.78±0.030
SCO	3.42±0.086	1.30±0.019	0.24±0.009	0.47±0.025	0.59±0.005	0.79±0.023
THA10	3.50±0.082	1.35±0.022	0.25±0.014	0.49±0.022	0.59±0.034	0.76±0.029
PSW10	3.32±0.064	1.26±0.026	0.24±0.010	0.49±0.006	0.59±0.019	0.75±0.007
PSW40	3.37±0.100	1.26±0.040	0.25±0.009	0.53±0.014	0.58±0.031	0.74±0.016
LPSW100	3.41±0.082	1.36±0.036	0.28±0.011	0.50±0.010	0.61±0.014	0.74±0.009
LPSW400	3.43±0.057	1.34±0.024	0.26±0.014	0.49±0.007	0.59±0.016	0.76±0.014

642 Relative organ weight (%) = (Organ weight/Body weight) x 100

643 All values are mean ± S.E.

644 [1)] CON, control; SCO, scopolamine (1 mg/kg B.W., i.p.); THA 10, tacrin (10 mg/kg B.W., p.o.); PSW 10, pig skin
645 water extracts (10 mg/kg B.W. p.o.) with SCO; PSW 40, pig skin water extracts (40 mg/kg B.W. p.o.) with SCO;
646 LPSW 100, low molecular weight pig skin water extracts (100 mg/kg B.W. p.o.) with SCO; LPSW 400, low
647 molecular weight pig skin water extracts (400 mg/kg B.W., p.o.) with SCO.
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651 **Table 4. Acetylcholine (ACh) content, acetylcholine esterase (AChE) activity, and**

652 **monoamine oxidase-B (MAO-B) activity in brains of mice fed PSW and LPSW**

Treatments[1)]	ACh contents (μM/mg protein)	AChE activity (μM/min/mg protein)	MAO-B activity (μM resorufin/min/mg protein)
CON	15.88±1.361[a]	0.46±0.018[b]	6.34±0.275[b]
SCO	11.28±0.937[c]	0.60±0.021[a]	12.37±0.440[a]
THA10	14.72±0.493[ab]	0.47±0.023[b]	7.42±2.628[b]
PSW10	12.08±1.139[bc]	0.53±0.029[ab]	10.01±1.354[ab]
PSW40	13.21±1.015[abc]	0.52±0.046[ab]	9.35±0.793[ab]
LPSW100	14.16±0.823[abc]	0.45±0.031[b]	7.68±1.490[b]
LPSW400	15.35±0.151[a]	0.44±0.030[b]	7.18±1.286[b]

653 All values are mean ± S.E.

654 [a-d] Means within same column with different letter differ significantly ($p < 0.05$).

655 [1)] CON, control; SCO, scopolamine (1 mg/kg B.W., i.p.); THA 10, tacrin (10 mg/kg B.W., p.o.); PSW 10, pig skin

656 water extracts (10 mg/kg B.W. p.o.) with SCO; PSW 40, pig skin water extracts (40 mg/kg B.W. p.o.) with SCO;

657 LPSW 100, low molecular weight pig skin water extracts (100 mg/kg B.W. p.o.) with SCO; LPSW 400, low

658 molecular weight pig skin water extracts (400 mg/kg B.W., p.o.) with SCO.

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