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Water-soluble derivative of propolis mitigates scopolamine-induced learning and memory impairment in mice☆

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ABSTRACT

The water-soluble derivative of propolis (WSDP) was prepared from fresh Chinese propolis. Its major constituents were identified by high performance liquid chromatography (HPLC) analysis. It has been reported that propolis possessed a broad spectrum of biological activities but including few studies on learning and memory by now. Thus, this study was aimed to investigate the effect of WSDP on scopolamine-induced learning and memory impairment in mice. WSDP (50 mg/kg, 100 mg/kg) was given by intragastric administration (i.g.) 40 min prior to the intraperitoneal (i.p.) injection of scopolamine (1 mg/kg).The effect on amnesia was investigated with both hidden-platform acquisition training and probe trial testing in Morris water maze test. The results from 100 mg/kg WSDP group showed significant mitigation scopolamine-induced amnesia in mice. Furthermore, WSDP's effect on the acetylcholinesterase (AChE) activity in the cerebral cortex and hippocampus was also assayed. As a result, WSDP (100 mg/kg) significantly inhibited AChE activity in the hippocampus of scopolamine-treated mice. These results indicated that WSDP may mitigate amnesia in vivo through inhibition of AChE activity in the hippocampus, which suggested propolis may have potential as a pharmaceutical of brain protection with elderly population for preventing Alzheimer's disease (AD) and other neurodegenerative diseases.

1. Introduction

Propolis is a resinous substance collected by honeybees from leaf buds and cracks in the bark of various plants. It has been used extensively in folk medicine in both the east and the west for centuries. Propolis has been reported to possess various biological activities, such as antibacterial (Kujumgiev et al., 1999; Kartal et al., 2003), antiviral (Amoros et al., 1994; Kujumgiev et al., 1999), anti-oxidation (Isla et al., 2001; Kumazawa et al., 2004), anti-inflammatory (Wang et al., 1993; Hu et al., 2005), anticancer (Kimoto et al., 2001; Sforcin, 2007), antifungal (Kujumgiev et al., 1999; Murad et al., 2002) properties. Some of the observed biological activities might be attributed to the identified chemical constituents that partially stem from its high content of flavonoids (Bankova et al., 2000).

Although propolis in the form of ethanolic extract was used for commercial pharmaceutical preparations usually, the water-soluble fractions and forms of natural propolis can be applied both orally and parenterally, and have a better resorption. So they are believed to possess an improved medical efficacy (Maximova et al., 1985; Koenig and Dustmann, 1986; Manolova et al., 1987). A pronounced ability of water-soluble derivative of propolis (WSDP) to prevent experimental viral, bacterial, fungal infections and antitumor was documented previously (Bankova et al., 1988; Dimov et al., 1991; Oršolić and Bašić, 2003, 2005). However, whether or not there is an effect of WSDP on amnesia remains unknown.

Alzheimer's disease (AD) is a neurodegenerative disease and causes memory loss and dementia, which mostly affects the elderly population (Francis et al., 1999). The pathophysiology of AD is complex including defective beta-amyloid (Aβ) protein metabolism, abnormalities of glutamatergic, adrenergic, serotonergic and dopaminergic neurotransmission, and the potential involvement of inflammatory, oxidative and hormonal pathways (Cutler and Sramek, 2001). Currently, the mainstay treatments for AD are acetylcholinesterase (AChE) inhibitors, which increase the availability of acetylcholine (ACh) at cholinergic synapses (Kang et al., 2005). Since the cholinesterase inhibitors confer only modest benefits, additional non-cholinergic AD therapies are urgently needed. Multipotent agents aiming at diverse targets are expected to act better than the single-

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target-aiming counterparts in the fight against AD. Natural products, especially flavonoids, have been given much attention (Ji and Zhang, 2006). More and more natural products have been used for the treatment of certain neurological diseases in oriental countries. For example, Kang et al. (2005) had reported that ESP-102, a standardized combined extract of *Angelica gigas*, *Saururus chinensis* and *Schizandra chinensis*, have some neuroprotective characteristics against neuronal cell death and cognitive impairments, and the study on EGb 761, a standardized extract of Ginkgo biloba, suggested that the improved cognitive functioning may be secondary to neuroprotective properties that buffer the animal from the harmful effects of stress (Warda et al., 2002).

Based on the knowledge of the broad pharmacology properties of propolis and the lack of studies on the learning and memory up to now, the present work was designed to observe the effects of WSDP on learning and memory with both hidden-platform acquisition training and probe trial testing in Morris water maze test. Morris water maze has been regarded as one of the most frequently used laboratory tools in spatial learning and memory of neurobiology and neuropharmacology research, typically consists of a series of spatial learning acquisition training and spatial accuracy memory in probe trial. Many methodological variations of the Morris water maze task have been and are being used by research groups in different applications (D'Hooge and De Deyn, 2001). Furthermore, as it has been well established that the hippocampal formation is essential for spatial learning (Morris et al., 1982), the AChE activity in the cerebral cortex and hippocampus was also analyzed in this study.

2. Materials and methods

2.1. Drugs and reagents

Caffeic acid, ferulic acid, *p*-coumaric acid, 5, 5′-dithio-bis-2-nitrobenzoic acid (DTNB), acetylcholine iodide and neostigmine were purchased from Sigma (USA). Luteolin, genistein, apigenin, kaempferol, chrysin, acacetin and quercetin were purchased from Shaanxi Huike Botanical Development Co., Ltd (Xi'an, China). L-arginine (L-Arg) was purchased from GL Biochem (Shanghai) Ltd (Shanghai, China). 3, 4-Dimethoxycinnamic acid, pinobanksin 3-acetate and tectochrysin were isolated from the ethanol extract of Chinese propolis and identified by mass spectrometric (MS) detection and ^{1}H and ^{13}C Nuclear Magnetic Resonance spectra (NMR). Scopolamine was purchased from Shanghai Hefeng drug manufacturer (China).

2.2. Animals

Adult male Kunming mice weighing 25–30 g were obtained from the Animal Center of Lanzhou University. All mice used in water maze test, following 1 week adaptation period, were housed under standard condition of temperature 25 °C, 12 h/12 h light dark cycles and fed with a standard laboratory diet and water. All animal procedures conform to the Provisions and General Recommendations of Chinese Experimental Animal Administration Legislation.

2.3. Preparation of WSDP

A WSDP was prepared by the method described by Nikolov et al. (1987), with some modification. Fresh propolis was purchased from the local market. Briefly, propolis (50.0 g) was cut into small pieces and extracted 3 times with 500 ml distilled water at room temperature, for 24 h each time. The extracts were filtered through a 0.45 μm filtration membrane. And then, the filter residues were extracted with petroleum ether (60–90 °C) under the condition of hot reflux for 3 h to make the waxes (15.15 g) out of propolis. Finally, the residues were re-extracted with ethanol (250 ml, 3 times, 6 h) under 80 °C. The extracts were filtered and evaporated under reduced pressure to dryness, giving 23.82 g black–brown product.

To make the WSDP, the ethanol extract of propolis (EEP, 10.0 g) was dissolved in 3% L-Arg water solution (1000 ml). The mixture was stirred (≤60 °C), centrifuged (4000 rmp, 5 min) and lyophilized to give 36.2 g black–brown powder. WSDP was dissolved in Normal Saline (NS) with concentration (6.25 mg/ml, 12.5 mg/ml).

2.4. HPLC analysis of the main components in WSDP

The main constituents in WSDP were identified and determined by HPLC with photodiode array (PDA) detection (Waters 600, USA) and mass spectrometric (MS) detection (Mariner, API, USA). WSDP was dissolved in distilled water and reference compounds were dissolved in ethanol (each 2 mg/ml), filtered with a 0.45 μm filter prior to the injection of 10 μl into the HPLC system, respectively. The mobile phase consisted of 0.1% acetic acid in methanol (A) and 0.1% acetic acid in water (B). The gradient was 10–100% A (0–90 min) at a flow rate of 0.5 ml/min. By using many reference compounds, we were able to identify the main components in WSDP.

2.5. Morris water maze test and administration

The Morris Water Maze Tracking System (TME, Chengdu, China) was used in the experiment. The Morris water maze test was performed by the method of Morris (1984) with minor modification. The Morris water maze is a white circular pool (80 cm in diameter and 45 cm in height) with a featureless inner surface. The pool was located at one end of a small rectangular room with a variety of visual cues, distal (on the wall) and proximal (on the rim of the pool). The pool was filled to a depth of 30 cm with water (23±2 °C) which was mixed with 1 L milk. The circular pool of water maze was divided into 4 quadrants: east, south, west and north. A white platform (9 cm in diameter and 29 cm in height) was centered in the northeast quadrant and submerged 1.5 cm below the water surface so that it was invisible at water level. The swimming pool was monitored by a video camera which connected to a video recorder and a computer. When we put the mouse colored with picric acid into the pool, the system can recognize the mouse and record its escape latency and swimming paths automatically. In the water maze tests, the day before the test was dedicated to swimming training for 120 s in the absence of the platform. In the following 4 days, the mice were given two trial sessions each day with an inter-trial interval of 60 min. And the platform was fixed in the same place in the 4 training days. Whether a mouse found or failed to find the platform within 120 s, it was maintained on the platform for 15 s. Latency to escape from the water maze (finding the submerged platform), the swim speed and distance of each mouse spent in the experiment were recorded by the computer program. As propolis had been considered with low toxicity and used as health-food/dietary supplement safely (Burdock, 1998), a visible platform test to assess sensorimotor and/or motivational deficits after WSDP treatment was not included. The probe trial was made by removing the platform and allowing each mouse to swim freely for 120 s inside the pool in the fifth day.

The mice were divided into 5 groups randomly, and each day given with NS, piracetam (100 mg/kg, dissolved in NS, i.g.), WSDP (50 mg/kg, 100 mg/kg, dissolved in NS, i.g.), or L-Arg (100 mg/kg, dissolved in NS, i.g.), respectively. 40 min later, amnesia was induced by scopolamine (1 mg/kg, dissolved in NS, i.p.). All mice were tested for spatial memory 20 min after the injection of scopolamine. Each drug was given once a day.

2.6. AChE assay

AChE activity was measured by the method of Ellman et al. (1961) modified by Kang et al. (2003). Mice were given NS or WSDP (100 mg/

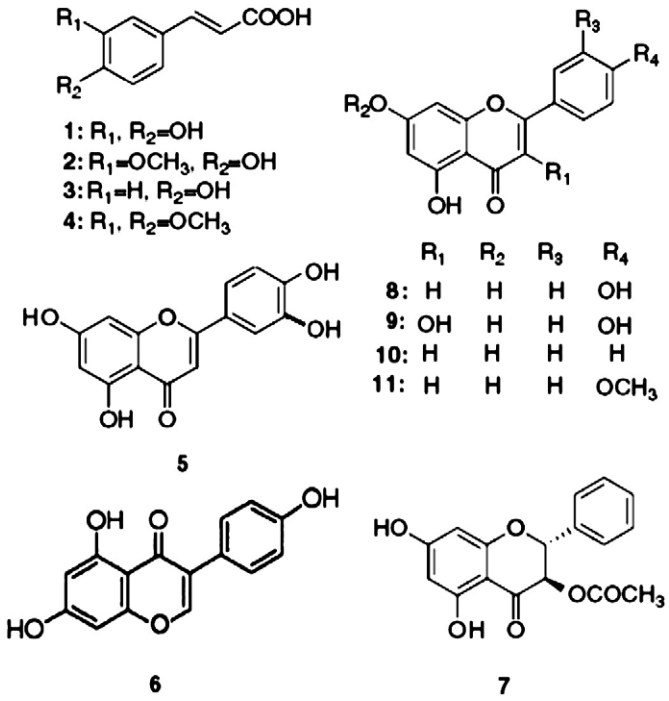

Fig. 1. Structures of the constituents identified from propolis. 1, caffeic acid; 2, ferulic acid; 3, *p*-coumaric acid; 4, 3,4-Dimethoxycinnamic acid; 5, luteolin; 6, genistein; 7, pinobanksin 3-acetate; 8, apigenin; 9, kaempferol; 10, chrysin; 11, acacetin.

kg). 40 min later, all mice were injected with scopolamine (1 mg/kg). The mice were decapitated 20 min after injection of scopolamine and the brains were removed. The cerebral cortex and hippocampus were dissected out. Each part of the brain tissue was rapidly homogenized, respectively, with sodium phosphate buffer (1 mM, pH 7.4). For the assay of AChE activity, a reaction mixture that contained sodium phosphate (1 mM, pH 8.0) 470 μl, 2% DTNB 167 μl and 33 μl of homogenate was incubated for 5 min at 37 °C. Then, acetylcholine iodide (2 mM) 280 μl was added to the reaction mixture. After incubation for 3 min at 37 °C, the reaction was terminated by adding 50 μl neostigmine (4 mM). Then, the absorbance was measured at 412 nm at room temperature. AChE activity was calculated as the optical density (OD) value per mg protein. Protein concentrations were determined by the Lowry method (Lowry et al., 1951) using bovine serum albumin as a standard.

2.7. Statistical analysis

All data were expressed as mean ± S.E.M. AChE activities (expressed as OD value per mg protein) for each tissue was analyzed by one-way ANOVA. Morris water maze latencies and swimming distance were analyzed by two-way ANOVA with the day as one variable and the treatment as a second. The data were considered to be significant statistically if the probability had a value of 0.05 or less.

3. Results

3.1. The major constituents of WSDP analyzed by HPLC

The major constituents of WSDP were identified by HPLC, by comparing the retention time with reference compounds. To identify each peak, WSDP was coeluted with reference compounds, respectively. The chemical structures of the major constituents are shown in Fig. 1. Fig. 2 shows the HPLC chromatograms of WSDP. The numbers 1–12 indicated the peaks identified by the HPLC analysis.

3.2. The effect of WSDP on hidden-platform acquisition training in Morris water maze test

The escape latency of finding a platform in the water maze was used as a measure for evaluating performance in tested mice. The escape latency was averaged for two trial sessions and for each mouse. The results were showed in Fig. 3A and B, in which all groups were compared with scopolamine-treated group in statistical analysis. The amnesia induced by scopolamine was significantly mitigated by a 100 mg/kg dose of WSDP ($n=9$, Fig. 3A, $P<0.01$), and the escape latency had decreased from 86.17 ± 11.62 s the first day to 42.93 ± 11.97 s the fourth day. However, a 50 mg/kg dose of WSDP did not significantly mitigate the amnesia induced by scopolamine ($n=10$, Fig. 3A, $P>0.05$). The positive control group (piracetam, 100 mg/kg, i.g.) significantly antagonized the effect of scopolamine ($n=10$, Fig. 3B,

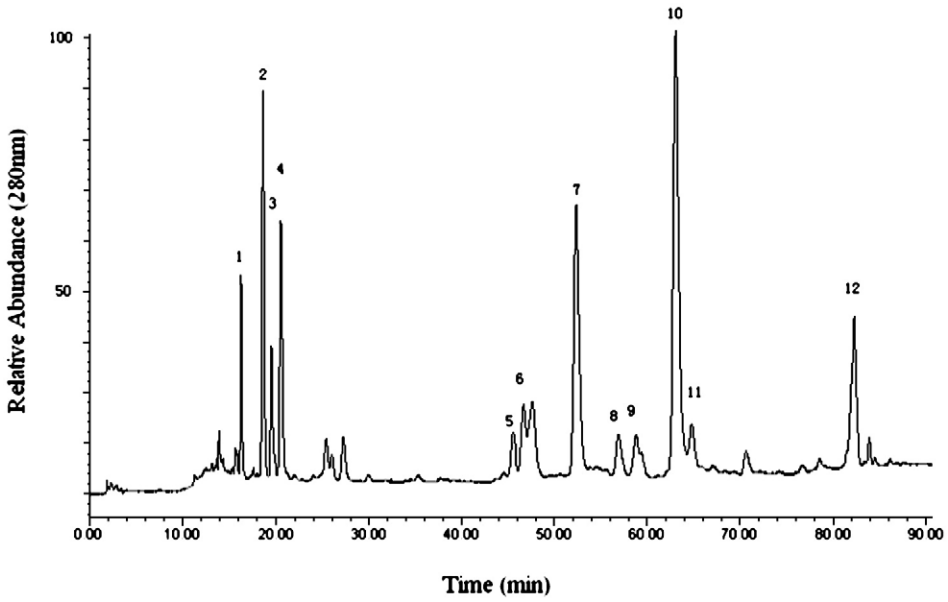

Fig. 2. HPLC chromatograms of WSDP. The numbers from 1 to 12 represent the different compounds. 1, caffeic acid; 2, ferulic acid; 3, *p*-coumaric acid; 4, 3,4-Dimethoxycinnamic acid; 5, luteolin; 6, genistein; 7, pinobanksin 3-acetate; 8, apigenin; 9, kaempferol; 10, chrysin; 11, acacetin;12, L-Arginine.

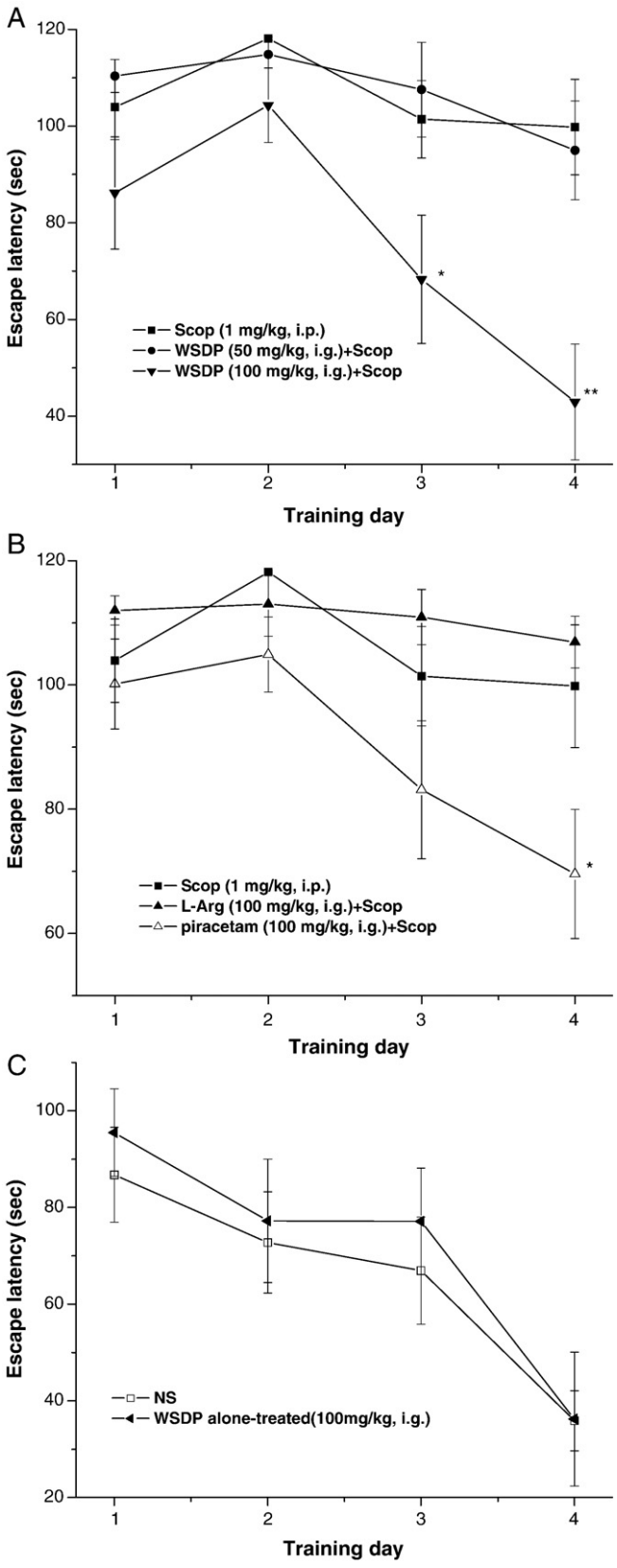

Fig. 3. The effect of WSDP on hidden-platform acquisition training in Morris water maze test. All values are expressed as the mean of two trails ± S.E.M. (A) Comparison of escape latency between WSDP-treated groups (n = 9–10) and scopolamine-treated group (Scop, n = 11) during four training days, $^{*}P<0.05$, $^{**}P<0.01$. (B) Comparison of escape latency between contrl groups (n = 10–11)and scopolamine-treated group (Scop, n = 11) during four training days, $^{*}P<0.05$. (C) Comparison of escape latency during four training days. There were no statistically significant difference between the NS alone-treated group (NS) and WSDP (100 mg/kg) alone-treated group (both n = 10, $P>0.05$).

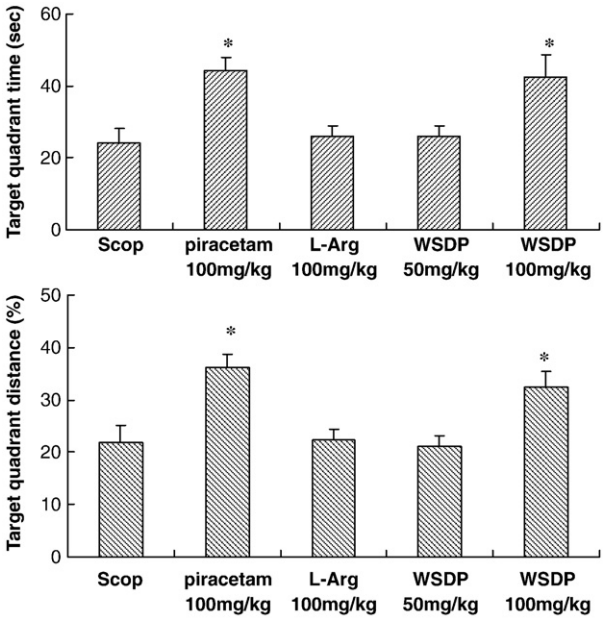

Fig. 4. The effect of WSDP on probe trial testing in Morris water maze test (n = 9–11). All values are expressed as the mean ± S.E.M. (A) Comparison of the time of swimming for each mouse spent in the target quadrant for probe trial within 120 s. (B) Comparison of the percent of the distance for each mouse spent in the target quadrant within 120 s. All treatments were compared with scopolamine-treated group (Scop), $^{*}P<0.05$.

$P<0.05$), the escape latency had decreased from 100.14 ± 7.2 s the first day to 69.58 ± 10.39 s the fourth day. In addition, L-Arg (100 mg/kg) did not significantly affect the amnesia induced by scopolamine (n = 11, Fig. 3B, $P>0.05$).

In order to determine whether or not WSDP has any effect on the spatial memory of normal mice, WSDP (100 mg/kg) was given to mice 40 min prior to water maze test. The results showed no statistically significant difference between the NS group and WSDP alone-treated group (both n = 10, Fig. 3C, $P>0.05$). Thus, WSDP treatment neither improves nor impairs on spatial learning and memory of normal mice. Besides, the data from the swim speeds of the mice in the 4 training days showed that there was no statistically significant difference among groups. All treatments were compared with scopolamine-treated group at the same trail ($P>0.05$, data not shown). Therefore, we could infer that the improvement in performance of WSDP-treated mice was independent of swim speed changes.

3.3. The effect of WSDP on probe trial testing in Morris water maze test

In the fifth day, the probe trail testing was performed by removing the platform and allowing each mouse to swim freely for 120 s inside the pool. The time and the distance of swimming for each mouse spent in the target quadrant (the northeast quadrant, where the platform was removed) were recorded. The average of time and the percentage of distance of swimming in target quadrant were calculated to make the comparison between groups. Compared with scopolamine-treated group, mice treated with WSDP and piracetam at a dose of 100 mg/kg showed significant more time and distance in the target quadrant (Fig. 4, $P<0.05$). Moreover, the data from the swim speeds of the mice in the test also showed no statistically significant difference (vs. scopolamine-treated group, $P>0.05$, data not shown).

3.4. The effect of WSDP on AChE activity

The results mentioned above showed the dose of 100 mg/kg was an efficacious dose for WSDP to mitigate the amnesia induced by scopolamine in the Morris water maze test. So we chose this dose in the assay of AChE activity. As a result, the WSDP (100 mg/kg)

Table 1
The effect of WSDP on AChE activity in cerebral cortex and hippocampus

Treatment	OD value/mg protein	
	Cerebral cortex	Hippocampus
Control	0.760±0.010	0.785±0.015
WSDP	0.756±0.029	0.654±0.015***

The values shown are the mean optical density/mg protein±S.E.M. Results differ significantly from values in same area of control: ***$P<0.001$. 6 mice were used in each treatment.

significantly inhibited AChE activity in hippocampus (Table 1, $P<0.001$), compared to control mice given with NS. However, in the cerebral cortex, there was no significant difference between the two groups on AChE activity.

4. Discussion

The effect of WSDP on scopolamine-induced learning and memory impairment was investigated in this study. The results demonstrate that WSDP at dose of 100 mg/kg can availably mitigate amnesia induced by scopolamine and inhibit the AChE activity in the hippocampus of mice. Piracetam is a clinic medicine that has been shown to improve cognitive performance in a number of animal model systems (Timothy et al., 2002). Compared with the positive control group of piracetam, the WSDP-treated group had the comparative effect on the mitigating amnesia in Morris water maze test. Furthermore, we also evaluated the role of the L-Arg containing in WSDP, and the data excluded its effect on the mitigating amnesia. This conclusion was consistent with the previous study (Bannerman et al., 1994). Thus, the pharmacological property of anti-amnesia in vivo could be attributed to the natural compounds of propolis in the mixture of WSDP.

As the composition of propolis may be different depending on geographical locations and plant sources (Marcucci, 1995; Nieva Moreno et al., 2000), we identified the main chemical components present in the WSDP (Fig. 2). 11 natural compounds including aromatic carboxylic acids and flavonoids were identified in the WSDP. Flavonoids show various biological and pharmacological activities, including anti-oxidative, anti-inflammatory, antitumor, and antiviral effects (Ross and Kasum, 2002). Recently, growing lines of evidence have suggested that flavonoids have neuroprotective effects in many models of neurodegenerative diseases in vitro and in vivo (Youdim et al., 2002; Lee et al., 2003; Dajas et al., 2003; Kang et al., 2004), but the mechanisms of flavonoids' neuroprotection are complicated and associated with multi-pharmacological effects. A recent study has examined the permeability of flavonoids and their known circulating metabolites across an in vitro model of the blood-brain barrier (BBB) (Youdim et al., 2003). And Youdim et al. (2004) also employed the rat in situ brain perfusion model to describe the BBB permeability of flavonoids. Therefore, the neuroprotective action of flavonoids could be attributed to selectively limited delivery to the CNS through BBB.

In this study, the results from AChE activity assay suggested that WSDP notably inhibited AChE activity in hippocampus of mice. Neurochemical studies suggested that acetylcholine (ACh) in neocortex and hippocampus predominantly affected the higher brain functions such as learning and memory (Francis et al., 1993). The most successful approach for AD treatment currently is to inactivate AChE, a molecule present in the synapse that rapidly cleaves ACh and prevents neuronal signaling (Cutler and Sramek, 2001). AChE inhibitors can enhance cognitive process in animals and humans, such as physostigmine, tacrine and velnacrine (Dawson et al., 1991; Braida et al., 1996; Rainer, 1997; Bejar et al., 1999). As we know, muscarinic cholinergic receptor antagonists as scopolamine have been shown to impair memory. Scopolamine has been used as a model in screening anti-amnesic drugs (Collerton, 1986; Kopelman and Corn, 1988). After i.p. injection of scopolamine, the cholinergic neurotransmission was blockaded, leading to increase of the AChE and impair cognition in mice (Levey, 1996). WSDP could notably inhibit AChE activity in hippocampus to mitigate amnesia. This effect may be owed to its complex components, especially flavonoids. Ji and Zhang (2006) had examined the structural requirements of flavonoids as inhibitors of AChE in vitro and indicated that substitutions of OH and OMe (or OH) at meta positions (positions 5 and 7) are beneficial to inhibiting AChE in a theoretical evaluation. There are abundant these kinds of flavonoids in WSDP, such as apigenin, kaempferol, acacetin. Some flavonoids may enter different brain regions after i.g. administration and exert their ability of inhibiting AChE. The selectivity of inhibitors for the AChE in the cortex and hippocampus, demonstrated in the current and previous studies (Enz et al., 1993; Weinstock et al., 1994; Bejar et al., 1999; Kang et al., 2003). Therefore, WSDP may have selective effect of inhibition on AChE in the cortex and hippocampus too. The damage to the hippocampus induced by scopolamine may be alleviated by WSDP, but the actual mechanism has not been clearly known. However, it is notable that the amnesia induced by scopolamine could be reversed by WSDP in the Morris water maze test.

Besides, neurodegenerative diseases such as AD are known to eventually be the result of neuronal cell death by many kinds of cause-induced excitotoxicity, oxidative stress, inflammation and apoptosis (Oyama et al., 1994). It has been shown that propolis has a wide range of biological activities, especially anti-oxidation and anti-inflammatory. Since the oxidation is promoted by free radicals, it probably could be slowed or perhaps even prevented by flavonoids (Hensley et al., 1994; Paladini et al., 1999). The main components of WSDP such as apigenin, kaempferol and luteolin had been reported that possessed neuroprotective action by their anti-oxidative capacity and free-radical scavenging activity (Wang et al., 2001; Kang et al., 2004). The Brazilian green propolis was reported possessing neuroprotective in PC12 cell culture and acted as an anti-oxidant against lipid peroxidation and free-radical production in vitro (Shimazawa et al., 2005). It has become clear in recent reports that flavonoids were beneficial in either preventing the onset or delaying the progression of AD through anti-inflammatory (Tang, 2005). Moreover, Tabet (2006) indicated that AChE inhibitors for the therapy of AD could be through anti-inflammatory. So it could be conferred that the activity of WSDP on mitigation of learning and memory impairment of mice maybe act partly through anti-oxidation and anti-inflammatory properties.

In summary, the anti-amnesia property of WSDP was proven in vivo, and this effect could be attributed to the inhibition of AChE by the high content of flavonoids in WSDP. Besides, some other biological mechanisms including anti-oxidation and anti-inflammatory could also contribute to this process. In traditional oriental medicine, it is conventional to use herbs in order to achieve a variety of treatment purposes simultaneously, or to enhance a single effect without causing severe side effects (Nishiyama et al., 1995). Hence, WSDP could be considered as a strategy to prevent or slow down the development of neurodegenerative diseases such as AD at an early stage.

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