

Neuroprotective effects of purslane herb aqueous extracts against D-galactose induced neurotoxicity

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Abstract

In order to evaluate mechanisms of natural plant purslane herb aqueous extracts (PHAS) for neuroprotective, we assessed neuroprotective effects of PHAS at doses of 2.5, 5 and 10 mg/(kg day) on SD mice injected daily with D-gal (50 mg/(kg day)) by behavioral tests. PHAS-fed mice showed higher activity upon induction by new environmental stimuli, lower anxiety and higher novelty-seeking behavior in the open field tasks, and significantly improved learning and memory ability in step-through compared with D-gal-treated mice. We further examined the mechanisms involved in neuroprotective effects of PHAS on mouse brain. PHAS significantly increased superoxide dismutase (SOD) activity and decreased the malondialdehyde (MDA) level. Meanwhile, PHAS also could up-regulate telomere lengths and telomerase activity in PHAS-fed groups. Furthermore, we examined the expression of p21^{waf1} and p53 mRNA and protein in mouse brain by western blot analysis and real-time RT-PCR. We found that p21^{waf1} was down-regulated by PHAS without changing the expression of p53. The results of this study suggested that the PHAS might be a primary target of p21^{waf1} and the neuroprotective effect of PHAS might be carried out through a p21^{waf1}-dependent and p53-independent pathway.

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The purslane herb, being a group of naturally occurring compounds widely distributed in most plants, has been used for thousands of years in traditional Chinese medicine and is one of the dietetic Chinese medicines which have been officially recognized as food and a Chinese medicine simultaneously [1]. The

anti-oxidative property of PHAS, being examined in different models, contains the following pharmacologically active substances including 28% flavonoids that are nearly exclusively flavonol-*O*-glycosides, 8% terpenoids (known as ginkgolides A, B, C and bilobalide), 6–12% organic acids, and >0.5% proanthocyanidins defined as flavonoid-based polymers [2,3]. Our research reveals that purslane herb is effective to be an antioxidant agent as well as nourishment of hepatic and brain in D-gal-induced mice [4]. Experimental evidence shows that PHAS have anti-oxidative effect in heart tissues in mice by increasing the activity of SOD [5]. Purslane herb pow-

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der also extends the life span of drosophila by regulating telomere length [6].

It is also known that D-gal has neurotoxic efficacy. Scientists in China are the first to report that injection of D-galactose can induce neurological impairment in rodents [7]. D-Gal induces behavioral impairment and some biochemical changes in the brain including increased production of ROS, reduces antioxidant enzyme activity, and attenuates immune. So D-gal-treated mouse serves as an induced neurotoxicity model has been used for neurotoxicity investigation and drug testing recently [8].

Experimental evidence accumulated during the last few years has shown that DNA oxidative damage can accelerate telomere shortening. The p53 and p21^{waf1} tumor suppressor are major determinant of the organism response to telomere dysfunction [9]. Several lines of evidence indicate that telomere shortening may lead to up-regulation of p53 and p21^{waf1} proteins as both oxidative and replicative induced telomere shortening can lead to cell aging [10–12].

The aim of the present study is to explore whether PHAS has neuroprotective effect on D-gal-induced neurotoxic mice and the regulation mechanism of PHAS on neuroprotective effect through a p21^{waf1}-dependent and p53-independent pathway in the brain.

1. Materials and methods

1.1. Preparation of aqueous extracts of purslane herb

The purslane herb was collected from the Department of Agent, Wuhan No. 1 Hospital, Wuhan, China (herbarium no. 220-155-06). The aqueous extract of the purslane herb were boiled in the traditional way. Briefly, herbs were minced and seeped in boiling water in the proportion of 1:10 (w/v) for 3 h. This was repeated two additional times for 3 h of boiling. After boiling, the resulting crude extract was filtered and the filtered extract was evaporated to dryness under reduced pressure at 40 °C and a yield of 24–28% (w/w) was obtained. The dried powder was kept at 4 °C for future use. All plant extracts were obtained from three independent extraction.

1.2. Preliminary phytochemical analysis

Plant extracts were screened for the presence of flavonoids and tannins, by using the methods previously described by Kilani et al. [13] and Tona et al. [14]. Two milligrams of each extract were separately dissolved in

2 ml of the appropriate solvent. The identification of major chemical groups was carried out by Thin Layer Chromatography (TLC) on silica gel 60 F 254 Merck (layer thickness 0.25 mm) as follows; for flavonoids, the TLC was developed in *n*-butanol/acetic acid/water (4:1:5) and the spots were visualized with 1% aluminium chloride in methanol under UV (366 nm); the test for tannins was carried out with FeCl₃. Each class of tannins gave a specific coloration.

1.3. Animals and treatment

Three-month-old SD male mice (The Laboratory Animal Center, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China) were randomly divided into five groups. Groups 2–5 of mice received daily subcutaneous injection of D-gal (Sigma–Aldrich, MO, USA) at dose of 50 mg/(kg day) for 8 weeks, and group 1 served as vehicle control with injection of PBS only. Then the groups 3–5 of D-gal-treated mice received PHAS at doses of 2.5, 5 and 10 mg/(kg day), respectively, for another 2 weeks. Meanwhile, another group of D-gal-treated mice and the group 1 control were given distilled water (dH₂O/0.1% Tween-80) without PHAS

1.4. Open field test

An open-field test apparatus was used for measuring the extent of locomotion and defecation within a fixed period of time. The apparatus consisted of a rectangular chamber (38 cm × 20 cm × 24 cm), with the field being illuminated with two 40-W light bulbs. The individual mice were placed in the middle of the chamber for each trial. After 1-min adaptation, the behavior of each mouse was recorded for 5 min by two observers 1 m away from the open-field area. During the trial intervals, the mice were returned to their home cage in the same room and the open field was wiped clean with a slightly damp cloth. The behavioral parameters were analysed as the following: (1) ambulation: the number of grids crossed in the arena during the observation period; (2) rearing: the number of times the mouse stands on its hind legs; (3) leaning: the number of times the mouse placed one or two forelimbs on the wall of the arena; (4) grooming: the number of times the mouse ‘washes’ itself by licking, wiping, combing or scratching of any part of the body.

1.5. Step-through test

The trough-shaped step-through passive avoidance apparatus consisted of an illuminated chamber attached

to a darkened chamber containing a metal floor that could deliver footshocks. The two compartments were separated by a guillotine door. Mice were placed in the dimly lit room containing the apparatus 0.5 h before training to acclimatize to the new environment. Each mouse was then placed individually into the illuminated chamber, facing away from the door to the dark chamber, and allowed to acclimatize for 1 min. When the mouse was observed to turn its body fully away from the dark chamber, the door was raised; when the next mouse turned fully toward the darkened chamber, the timer was started. An initial time measure was from the time that the mouse faced the opened darkened chamber to the time that the mouse fully entered, with all four paws, the dark chamber. As soon as the mouse entered the dark chamber the door was slid back into place, triggering a mild footshock. The mouse was then immediately removed from the chamber and returned to its home cage. The retention test was conducted 24 h later with the mouse again being placed in the illuminated chamber and subjected to the same protocol described above in the absence of footshock. The upper time limit was set at 300 s.

1.6. SOD assay

SOD activity was measured according to the method described by McCord and Fridowich [15]. Solution A was prepared by mixing 100 ml of 50 mM PBS (pH 7.4) containing 0.1 mM EDTA and 2 μ mol of cytochrome *c* with 10 ml of 0.001N NaOH solution containing 5 μ mol of xanthine. Solution B included 0.2 U xanthine oxidase/ml and 0.1 mM EDTA. Fifty microlitres of a tissue supernatant were mixed with 2.9 ml of solution A and the reaction was started by adding 50 μ l of solution B. Change in absorbance at 550 nm was monitored. A blank was run by substituting 50 μ l ultra pure water for the supernatant. SOD levels were expressed as U/mg protein with reference to the activity of a standard curve of bovine copper–zinc SOD under the same conditions.

1.7. MDA assay

The level of MDA in brain tissue was determined using the method of Uchiyama and Mihara [16]. Half a milliliter of homogenate was mixed with 3 ml of H₃PO₄ solution (1%, v/v) followed by addition of 1 ml of thiobarbituric acid solution (0.67%, w/v). Then the mixture was heated in a water bath for 45 min. The colored complex was extracted into *n*-butanol, and the absorption at 532 nm was measured using tetramethoxypropane as

standard. MDA levels were expressed as nmol per mg of protein.

1.8. Telomere length measurement

Determination of telomere restriction fragment length Southern analysis of telomere restriction fragment (TRF) length. Genomic DNA was isolated from 50 mg mice brain tissue using DNA isolation kit (Qiagen, Valencia, CA, USA) and quantitated by UV spectrophotometry. Two micrograms of DNA were digested with restriction endonucleases RsaI and HinfI. The DNA digests were electrophoresed through 0.8% agarose and transferred to nylon membranes by capillary transfer in 20 \times SSC as described. After UV crosslinking (1200 μ J), the membranes were hybridized with a 3'-digoxigenin oligonucleotide probe with the sequence (CCCTAA)₃. After washing to remove unbound probe, an alkaline phosphatase-conjugated anti-digoxigenin antibody (Roche Applied Science, Indianapolis, IN, USA) was used for immunodetection of bound probe, followed by CDP Star chemiluminescence substrate (Roche Applied Science, USA). Blots were exposed to X-ray film for 10–60 s. Mean TRF length and percentage photo-stimulated luminescence were determined from densitometric analysis of digital images of exposed films as described. Measurements of TRF length were performed in duplicate for each brain.

1.9. Detection of telomerase activity

The telomerase activity was measured using a PCR-TRAP ELISA kit (Roche Applied Science, USA) according to the manufacturer's description with some modifications. For the TRAP reaction, 2 μ g protein was added to 25 μ l of reaction mixture with the appropriate amount of sterile water to create a final volume of 50 μ l. Hybridization and the ELISA reaction were carried out following the manufacturer's instructions.

1.10. Western blotting

Samples (brain tissue) were extracted at 4 °C for 30 min. After centrifugation at 12,000 rpm for 20 min, the supernatant were obtained as cell lysate and electrophoresed on 10% SDS-PAGE and transferred to a PVDF membrane. The membrane was allowed to react with anti- β -actin polyclonal, anti-p21, p53 antibody (Santa Cruz Biotechnology, Delaware City, Santa Cruz). Specific antibodies were detected with a chemiluminescence kit (Sigma-Aldrich China Inc. Shanghai, China)

according to the supplier's manual. Chemiluminescence was detected by exposure to X-ray film.

1.11. Quantification of p21 and p53 mRNA by real-time PCR

The mRNA levels for p21 and p53 were determined by real-time RT-PCR using QuantiTect SYBR Green RT-PCR kit (Qiagen Ltd., Crawley, UK) on a LightCycler (Roche Diagnostics Ltd., Lewes, UK). Total RNA was isolated from brain tissue of groups 1–5 with the Trizol reagent (GIBCO–BRL, Invitrogen Corp., Carlsbad, CA) according to the manufacturer's instruction. Primers for p21^{waf1} (forward: CCCGAGAACGGTG-GAACT and reverse: GTGGGCACTTCAGGGTTT) and p53 (forward: CTCAAAAACTTACCAGGGC and reverse: CACCACGCTGTGGCGAAAAGTCTG) were designed using primer premier 5 (Perkin-Elmer, Foster City, CA, USA) and synthesized by Shanghai Gene Core Bio Technologies Co, Ltd. (Shanghai, China). RT-PCRs were performed in 20 μ l reactions using 10 pmol of primers. Reverse transcription was carried out at 50 °C for 20 min, and cDNA was amplified in 36–46 cycles: 94 °C for 15 s, 57 °C for 20 s, and 72 °C for 5 s. Negative controls with no template were performed for each reaction. The relative quantity of gene expression was calculated according to the manufacturer's recommendations. Mice β -actin (Applied Biosystems China, Beijing, China) was used as an internal control to calculate the relative abundance of this gene.

1.12. Statistical analysis

All data in the text and figures were presented as means \pm standard deviation (means \pm S.D.). Statistical analysis was carried out using Student's *t*-test.

2. Results

2.1. Phytochemical study and metabolites content

The phytochemical study of PHAS showed the presence of various quantities of total polyphenolic compounds, tannins and particularly flavonoids. The metabolites contents and variety of the tested extracts are presented in Table 1.

2.2. Effects of PHAS on the behavior of D-gal-treated mice

Data presented in Fig. 1A indicate that the group of mice received daily subcutaneous injection of D-

Table 1

Total polyphenols, total flavonoids and tannins contents of PHAS vary each extraction

%Yield and metabolites	Extracts		
	First time (%)	Second time (%)	Third time (%)
%Yield ^a	24.8	26.7	27.2
Polyphenols ^b	42.3	39.5	40.2
Flavonoids ^b	22.8	27.2	25.9
Tannins ^b	34.9	33.3	33.9

^a %Yield to the dried herbs.

^b %Dry-weight basis.

gal at doses of 50 mg/kg (D-gal model) reduced their activities of crossing ($F(4,40) = 3.12$, $P < 0.05$), rearing/leaning ($F(4,40) = 2.06$, $P < 0.05$) and grooming activity ($F(4,40) = 1.24$, $P < 0.05$) as compared with the control group. This result suggested impairment of the exploration activities in mice treated with D-gal. When three groups of D-gal-induced neurotoxic mice were given 2.5, 5 and 10 mg/(kg day) of PHAS, respectively, for another 2 weeks, their activities of rearing/leaning ($P < 0.05$) and grooming ($P < 0.05$) were significantly increased.

The step-through latency in the 24 h-retention trial was significantly decreased in the D-gal-treated mice, as

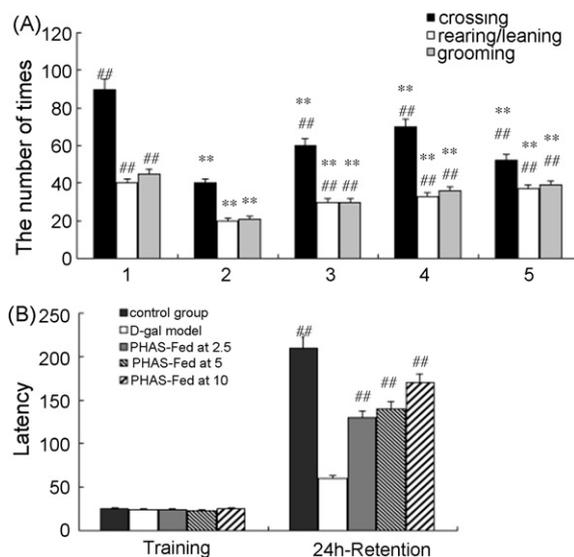


Fig. 1. Effects of PHAS on the behavior of D-gal-treated mice. The locomotor and exploratory behavior of D-gal-treated mice fed with PHAS at different doses or control group (A). The step-through latency of D-gal-treated mice fed with PHAS at different doses or control group (B). All data were obtained from three independent experiments. Lanes 1–5 are untreated control mice, D-gal model. PHAS-fed at 2.5, 5 and 10 mg/(kg day), respectively. Error bars represent means \pm S.E.M. Significantly different from the corresponding control (** $P < 0.05$ vs. control; ### $P < 0.05$ vs. D-gal model).

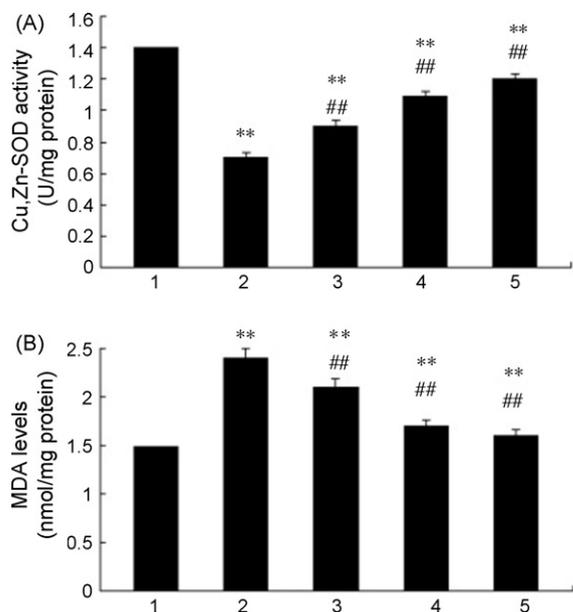


Fig. 2. Effects of PHAS on the SOD activity and the MDA levels in D-gal-treated the brain of mice. The effects of PHAS on the activity changes of Cu, Zn-SOD (A). The effects of PHAS on the changes of MDA level (B). Lanes 1–5 are untreated control mice, D-gal model. PHAS-fed at 2.5, 5 and 10 mg/(kg day), respectively. All data were obtained from three independent experiments. Error bars represent means \pm S.E.M. Significantly different from the corresponding control (** $P < 0.05$ vs. control; ## $P < 0.05$ vs. D-gal model).

compared to the control (Fig. 1B). In the 24 h-retention trial the latency of the D-gal-treated mice received daily 2.5, 5 and 10 mg/(kg day) PHAS for 2 weeks were significantly increased versus D-gal model.

2.3. Effects of PHAS on the SOD activity and the MDA levels in D-gal-treated the brain of mice

The D-gal treatment resulted in significant decreases in Cu, Zn-SOD activity in the brain ($P < 0.05$) (Fig. 2A). The D-gal-treated mice received PHAS showed a significant attenuated in the Cu, Zn-SOD activity; higher dose of PHAS had greater effect than 2.5 mg/(kg day).

Fig. 2B described the levels of MDA in the brain of five groups of mice. Compared with the vehicle control, D-gal-treated group showed a significant increase in MDA level ($P < 0.05$). This increase in MDA was also attenuated in the brain of PHAS-fed mice. As compared with the D-gal-treated group, a higher dosage of PHAS (5 and 10 mg/(kg day)) exerted more significant difference on MDA level than the PHAS at the dose of 2.5 mg/(kg day) ($P < 0.05$) in the brain of mice.

2.4. Effects of PHAS on telomere length and telomerase activity in D-gal-treated the brain of mice

TRF length was determined using pulse gel electrophoresis followed by Southern blot hybridization with telomere-specific probes. Average telomere lengths were shown in Fig. 3A. Telomere length assay revealed that PHAS-fed groups were longer than those in the controls and D-gal model groups. PHAS-fed group of different concentrations led to resulted in the significant increment of the mean telomere length (Fig. 3B, $P < 0.05$).

We then again evaluated the effect of PHAS on telomerase activity. Our data suggested that PHAS could up-regulate telomerase activity in PHAS-fed groups. Telomerase activity were shown in Fig. 3C. It revealed that PHAS-fed groups had higher than those in the controls and D-gal model groups. PHAS-fed group of different concentrations led to resulted in the significant increment of the telomerase activity.

2.5. Effect of PHAS on p21^{waf1} and p53 expression in D-gal-treated the brain of mice

The p21^{waf1} exerted an inhibitory effect on cyclin-dependent kinase and mediated cell growth arrest. We therefore assumed PHAS treatment could cause an reducing expression of p21^{waf1}, western blot analysis and real-time RT-PCR showed that in each group brain, a sustained reduce in expression of p21 was observed after treatment with different concentration. PHAS-fed of different concentrations, resulted in the significant attenuation of expression of p21^{waf1} mRNA and protein ($P < 0.05$) (Fig. 4A–C).

As expression of p21^{waf1} could be regulated through both the p53-dependent and the p53-independent pathways, the brain p53 mRNA and protein level in each group was detected by western blot analysis and real-time RT-PCR. As shown in Fig. 4A–C, there was no change of p53 in treatment process.

These data indicated that PHAS might inhibit the expression of p21^{waf1}. However, there were no changes of p53 in each group with different treatments. Taken together, it suggested that PHAS played an active effect in up-regulate telomerase activity and telomere length through a p21^{waf1}-dependent and a p53-independent pathway.

3. Discussion

The purslane herb is an annual plant which grows in the oriental and its several pharmacological effects such as diuretic, anti-ascorbic, antipyretic, anti-asthma,

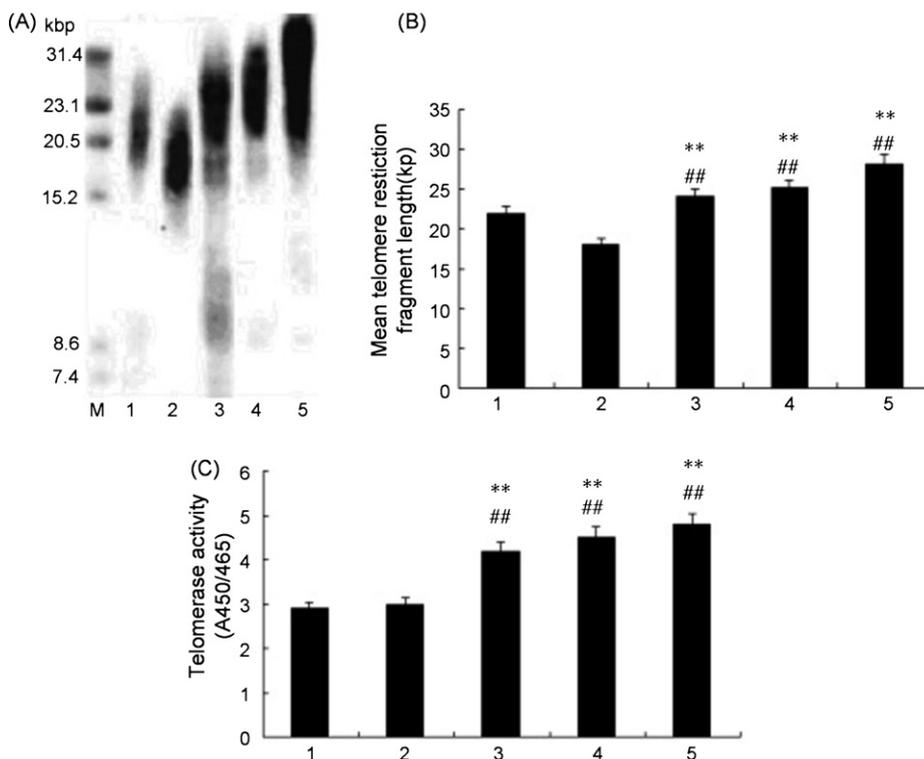


Fig. 3. Mean telomere restriction fragment length in control and PHAS-fed groups brains were determined by Southern analysis as described in Section 1. Representative Southern blot of brain telomere restriction fragments. Locations of the base pair markers on the DNA ladder are indicated along the left side (A). Significant difference was observed between the mean telomere lengths of the control and PHAS-fed groups brains (B). Representative the concentration-course analysis of telomerase activity, each groups brains tissue were mixed with 1 ml TBA solution for preparation of protein extract and 1 μ g protein was subjected to TRAP assay. After hybridization and ELISA procedure, the absorbance of the samples at 450 nm (with a reference wavelength of approximately 655 nm) was measured (C). Lanes 1–5 are untreated control mice, D-gal model. PHAS-fed at 10, 5 and 2.5 mg/(kg day), respectively. All data were obtained from three independent experiments. Error bars represent means \pm S.E.M. Significantly different from the corresponding control (** $P < 0.05$ vs. control; ## $P < 0.05$ vs. D-gal model).

anti-inflammatory, and anti-tussive effect [17]. In the present study, we observed that PHAS was effective antioxidative agent which could, to some extent, reverse D-gal-induced neurotoxicity resulted from oxidative damages by regulating the ROS level in the brain of mice.

In behavioral tests, we found that, in consistent with previous reports, D-gal could induce remarkable learning and memory impairment in mice. The mice took longer time to find the platform and made more mistakes in step-through test (Fig. 1). Meanwhile, PHAS-fed mice showed an enhanced active behavioral response to the open field which could be interpreted either as lower anxiety and high motivation to explore or as higher sensitivity towards new stimuli compared with the D-gal-treated mice (Fig. 1A). Moreover, PHAS could also reverse the D-gal-induced memory impairment in the step-through tasks (Fig. 1B).

The activity of SOD was reported to decrease with age due to the irreversible inactivation by its dismutation product, hydrogen peroxide or over-glycation product.

MDA is a major biomarker of lipid peroxides [18]. Long-term administration of D-gal-induced changes of these redox-related biomarkers in mice, including decrease in SOD increase of MDA level. These alterations were considered to play important roles in learning and memory deficits. In the present study, we found that PHAS could significantly increase the activities of anti-oxidative enzymes such as SOD and decrease the production of MDA level in the aging mice brain (Fig. 2). This indicated that PHAS, to some extent, could attenuate the oxidative injury induced by D-galactose. In other words, the protection of PHAS against oxidative stress to brain may be involved in the mechanism of its action to ameliorate the impairments of learning and memory.

It is well known that DNA oxidative damage can accelerate telomere shortening and contribute to the aging process [19]. The correlation between aging and telomere erosion led to the formulation of the telomere hypothesis, which proposes that the gradual reduction in telomere length cause by successive rounds of DNA

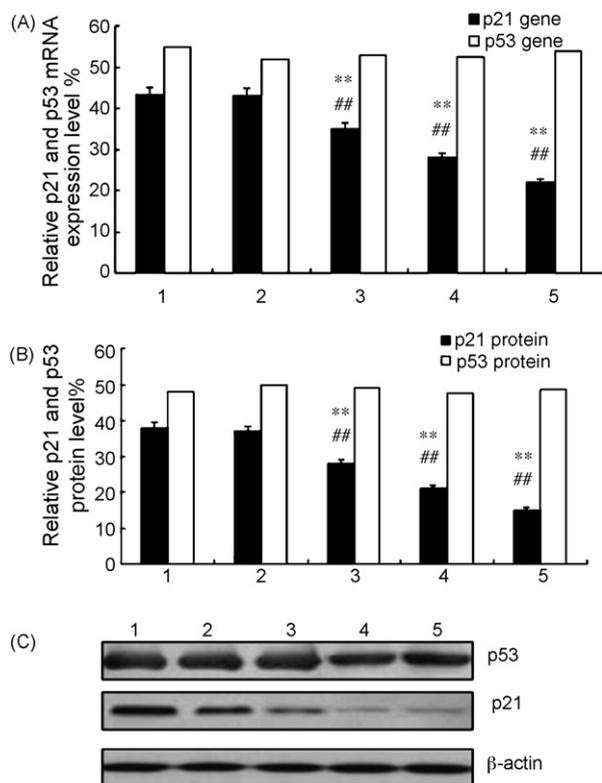


Fig. 4. Effect of PHAS on p21^{waf1} and p53 gene expression. The p21^{waf1} and p53 mRNA expression in brain were detected by RT-PCR with different treatments (A). Protein expression of p21^{waf1} and p53 in brain were quantified by densitometric analysis (B). Typical Western blot results for p21^{waf1} and p53 protein (C). Lanes 1–5 are untreated control mice, D-gal model. PHAS-fed at 10, 5 and 2.5 mg/(kg day), respectively. All data were obtained from three independent experiments. Error bars represent means \pm S.E.M. Significantly different from the corresponding control (** $P < 0.05$ vs. control; ## $P < 0.05$ vs. D-gal model).

replication and cell division causes the length of one or more telomeres length to fall below a certain threshold, resulting in the initiation of aging [20]. Therefore, we analysed telomere length and telomerase activity in brain tissue of each group mice PHAS, Fig. 3 revealed that the telomere length and telomerase activity in the brain of PHAS-fed mice were significantly longer and higher than those in D-gal-treated mice. Therefore, PHAS can significantly increase the telomere length and telomerase activity in D-gal-treated mice.

It is demonstrated that the p53 tumor suppressor is a major determinant of the organism response to telomere dysfunction [21,22]. Previous work has shown that telomere dysfunction was sensed in a manner similar to DNA double-strand breaks, with activation of p53 effecting either senescence or apoptosis. The p53 was showed to activate p21^{waf1} transcription and it was reported

that expression of p21^{waf1} can be regulated through both the p53-dependent and the p53-independent pathways [23], therefore we assumed PHAS could affect p53 expression and subsequently induce p21^{waf1} expression. To address this question, we detected p21^{waf1} and p53 mRNA and protein level in brain tissue of each group mice PHAS treated with different concentration (Fig. 4). Our data showed p53 could be detected but not changed with treatment. However, expression of p21^{waf1} mRNA and protein significantly reduced. This suggested that PHAS down-regulated p21^{waf1} expression be a p53-independent pathway. We therefore proposed that p21^{waf1} was a primary target of PHAS and played an important role in mediating PHAS-protected telomere length effect on D-gal-induced mice.

In summary, our results showed that PHAS could reverse the chronic D-gal-induced neurotoxicity. PHAS-fed mice showed an improved performance in all behavioral tests. Based on our data, we concluded that the mechanism of the neuroprotective effects of PHAS on D-gal-induced neurotoxicity may be: (i) increasing the antioxidant enzyme activities, for example, SOD, and protecting against lipid peroxidation and (ii) reducing the expression of p21^{waf1} mRNA and prolonging telomere length. However, the up-regulation of telomerase activity combined with the down-regulation of p21^{waf1} was a relatively dangerous position for cell cycle and maybe permissive for malignant transformation. Further research is needed to understand how the p21^{waf1} pathway is attenuated in normal cell and explore the mechanism that PHAS regulates p21^{waf1} expression either in a direct way or indirectly by another cascade.

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