A Journal of the Bangladesh Pharmacological Society (BDPS)
Journal homepage: www.banglajol.info
Abstracted/indexed in Academic Search Complete, Asia Journals Online, Bangladesh Journals Online, Biological Abstracts, BIOSIS Previews, CAB Abstracts, Current Abstracts, Directory of Open Access Journals, EMBASE/Excerpta Medica, Google Scholar, HINARI (WHO), International Pharmaceutical Abstracts, Open J-gate, Science Citation Index Expanded, SCOPUS and Social Sciences Citation Index;
Bangladesh J Pharmacol 2016; 11: S168-S176
ISSN: 1991-0088

Anti-aging activity of Radix polygoni multiflori preparata in aging mice model induced by D-galactose by bioassay method

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Article Info

Received: 11 January 2016
Accepted: 1 March 2016
Available Online: 11 March 2016

DOI: 10.3329/bjp.v11iS1.27010

Cite this article:
Huo JY, Shen HC, Wang SM, Chen ZK, Liang SW. Anti-aging activity of Radix polygoni multiflori preparata in aging mice model induced by D-galactose by bioassay method. Bangladesh J Pharmacol. 2016; 11: S168-S176.

Abstract

Anti-aging effect of Radix polygoni multiflori preparata in aging mice model was examined by bioassay method and explore new model of quality control of Traditional Chinese Medicine. Model of aging mice induced by D-galactose were used. The activity of senescence associated molecular markers such as superoxide dismutase, malondialdehyde and glutathione peroxidase in serum or homogenate were determined to evaluate the anti-aging activity. The results showed that the polysaccharide content in the 0.4, 0.8 g/mL decoction were 80.1, 160.2 mg/mL respectively. The HPLC results showed that stibene glucoside content in the 0.4 and 0.8 g/mL decoction were 404 and 808 mg/mL respectively. Experimental results showed that 10 week-old mice were the most sensitive to SOD activity in homogenate. In conclusion, Radix polygoni multiflori preparata has anti-aging effect probably by removing ROS.

Introduction

According to the aging theory, the production of reaction oxygen species (ROS) or free radicals could injure cells and tissues paralleled by malfunction of many systems, and eventually lead to aging and cell death (Goldberg et al., 2014).

Glycation is a non-enzymatically driven reaction between free amine groups, and the (Maillard) reaction eventually leads to the formation of advanced glycation end products (AGEs). Glycation is thought to play an important role in aging and the AGEs exacerbate and accelerate the aging process, because AGEs and their precursors usually contain reactive carbony groups generated by ROS (Tian et al., 2005; Semba et al., 2005; Chen et al., 2012; Stadtman, 1992). ROS bind to polyunsaturated lipids, forming malondialdehyde (MDA) which is a reactive aldehyde and one of many reactive electrophile species that causes toxic stress in cells similarly to AGEs (Gil et al., 2002).

Superoxide dismutase (SOD), the prime anti-oxidant enzyme converting superoxide anion to oxygen and hydrogen and hyrogen peroxide (H_2O_2), reduces reactive oxygen species formation associated with oxidative, aging diminishes the overall activity of SOD and increases sensitivity to oxidative stress. Glutathione peroxidase (GSH-PX) can neutralize a broad range of peoxides and convert H_2O_2 to water and oxygen in the presence of glutathione. Therefore, the level of MDA and the activity of SOD and GSH-PX could be used as markers of the aging process (Rasheed et al., 2014; Brigelius Flohe, 1999; Bilgili et al., 2013; Prie et al., 2015).

Radix polygoni multiflori (He shou wu) is the dried root tuber of polygoni multiflorum Thunb. (Fam. polygonaceae). It becomes Radix polygoni multiflori preparata after being processed with black soybean juice according to the processing methods recorded in

the Chinese Pharmacopoeia (Liang et al., 2010).

Radix polygoni multiflori preparata is used to replenish the liver and kidney with vital essence and blood, blacken the hair and strengthen the tendons and bones according to traditional Chinese medicine theory. Radix polygoni multiflori preparata is also used to lengthen life in some old traditional Chinese literature (Yao et al., 1984). The failure of liver and kidney is mainly reflected in a series of physical aging phenomenon.

The present study demonstrated that Raix polygoni multiflori preparata possessed anti-aging effect by removing free radical, repairing injured gene, enhancing the body's immune function, delaying cell aging or other mechanism (Tan, 2004).

Bioassay method is to evaluate biological activity of organism, including the whole animal, isolated organs, tissues, cells and so on. Specific experimental designs were applied to compare the response between samples and equivalent standards to determine the potency, biological activity or toxicity of samples (National Pharmacopoeia Committee, 2010). It is applied for the medicine that the constituents are complex or the contents could not be tested by physical and chemical methods or the clinical bioactivity could not be reflected by the physical and chemical measurement (Chen and Lai, 2001). Bioassay has many advantages and huge potential on the quality control and evaluation of traditional Chinese medicine due to the complexity of traditional Chinese medicine (Wang et al., 2009). It has become a research hotspot in recent years, such as the exploration of the bioassay method of Leonurus, Forsythia and other Chinese medicinal materials (Yang et al., 2002a; Yang et al., 2002b; Yang and Wang, 2002; Zhao et al., 2008; Ma et al., 2010). So, bioassay method was used to explore new model in the quality control of TCM, and realize more comprehensive and reasonable quality standards of TCM by the combination of determination of biological activity of TCM and modern analysis technology.

No recent literature demonstrated the anti-aging effect of Radix polygoni multiflori preparata by bioassay method.

Materials and Methods

Instrumentation

An UV-2450 Shimadzu ultraviolet spectrophotometer (Japan) was used to measure the absorbance. An Agilent 1260 series HPLC-DAD system comprising a vacuum degasser, binary pump, autosampler, thermostated column compartment and DAD (Agilent, USA) was used for the content determination of stibene glucoside, emodin and emodin monomethyl. Separation was performed at room temperature on an Phenomenex C18 analytical column (250 mm × 4.6 mm i.d., 5 mm). The mobile phase system for content determination of stibene glucoside was acetonitrile-water (25:75) at 20 min at a flow rate of 1 mL/min. The injection volume was 20 µL and detection was performed at 320 nm. The mobile phase system for content determination of emodin and emodin monomethyl ether was methanol :0.1% phosphoric acid solution (80:20) at 30 min at a flow rate of 1 mL/min. The injection volume was 20 µL and detection was performed at 254 nm. MS2 mini oscillator (IKA, German), ZK-82A vacuum drying oven (Shanghai experimental instrument factory), Mettler AE240 electronic analytical balance (Switzerland), BSZZ4S type electronic analytical balance (Beijing Sartorius Instrument System Co., Ltd), KQ-100 type ultrasonic cleaning machine (Kunshan Ultrasonic Instrument Co., Ltd), TGL-10B flying pigeon high-speed bench centrifuge (Shanghai Anting Scientific Instrument Factory), DK-S26 electrothermal constant warm bath pot (Shanghai Jing Hong Laboratory Instrument Co., Ltd), RE-52A type rotary evaporation instrument (Shanghai Yarong Biochemical Instrument Factory), FN 202-2 type electric heated drying oven (Changsha Instrument and Meter Plant), pipetting gun (Socorex, Switzerland) were used for the preparation of vitamin E solution, stibene glucoside, emodin and emodin monomethyl solution and the extraction of Radix polygoni multiflori preparata.

Chemicals and reagents

SOD kit was purchased from Nanjing Jiancheng Biological Engineering Research Institute. D-galactose was purchased from Shanghai Pharmaceutical Factory. Vitamin E was provided by Guangzhou Tianzhi Biological Technology Co., Ltd. Reference compounds of D-anhydrous glucose, stibene glucoside, emodin and emodin monomethyl ether (all with purities >98%) were purchased from national institute for the control of Pharmaceutical and Biological Products, China (Batch No. 110833-201205, 110844-201109, 110756-200910, and 110758-201013 respectively). Deonized water was generated from a Milli-Q water purification system (Millipore, USA), HPLC grade methanol and acetonitrile were provided by Tedia Company Inc., phosphoric acid and other reagents were analytical pure.

Plants

Radix polygoni multiflori preparata (producted in Deqing, Guangdong Province, batch number 090310) was purchased from Guangzhou Zhixin Medicine Health Co. Ltd, and authenticated macroscopically by Prof. Li Shuyuan, Guangdong Pharmaceutical University. Radix polygoni multiflori preparata purchased was identified, examined and determined according to the Chinese pharmacopoeia (Version 2010).

Preparation of D-galactose solution, vitamin E solution and plant decoction

D-galactose was accurately weighed and dissolved in saline solution to produce 15 mg/mL D-galactose solution. Vitamin E was accurately weighed, dissolved and diluted with physiological saline to produce 3.6 mg/mL as high dose. The decoction Radix polygoni multiflori preparation was produced according to the traditional clinical decoction method documented in literature as following: Two copies of 40 g of crude drugs through a 50 mesh sieve were boiled with 400 mL distilled water 2 times for 1 hour each time respectively. The decoction was combined, filtered, concentrated and dissolved with distilled water in 50 mL, 100 mL volumetric flask respectively to produce 0.8, 0.4 g/mL decoction and the concentration ratio was 2:1.

Content determination of Radix polygoni multiflori preparata decoction

The content determination of polysaccharide, stibene glucoside, emodin and emodin monomethyl ether were contained in the content determination of Radix polygoni multiflori preparata decoction. The content determination of polysaccharide was performed by phenol-sulfuric acid colorimetric method. D-anhydrous glucose reference substance was accurately weighed and diluted with distilled water to produce 0.01 to 0.06 mg/mL standard solution to do the linear test. 5 mL continued filter decoction was taken to 500 mL volumetric flask and diluted with distilled water as the test solution. 2 mL standard solution and the test solution were respectively added 1 mL 5% phenol solution and 5 mL sulfuric acid in the test tube. The solution in the test tube was vibrated and boiled in water bath for 15 min. It was determined under 490 nm according to the UV spectrophotometry method.

The content determination of stibene glucoside, emodin and emodin monomethyl were performed by HPLC according to the methods recorded in Chinese pharmacopoeia. Stibene glucoside reference substance was accurately weighed and dissolved in 50% alcohol to produce 0.2 mg/mL standard solution. Emodin and emodin monomethyl reference substance were accurately weighed and dissolved in methanol to produce 80 μg/mL emodin and 40 μg/mL emodin monomethyl mixed reference substance solution. 5 mL continued filter decoction was taken to 500 mL volumetric flask and diluted with 80% methanol as the test solution.

Animal handling

The KM mice (Certification number: SCXK Cantonese 2013020) of both sexes were purchased from Experimental Animal Center of Guangzhou University of Chinese Medicine. All mice were kept in plastic cages with stainless steel box and plastic water bottle at a barrier system with regulated temperature (23 ± 2°C) and humidity (60 ± 5%), and on 14 hours light/10 hours dark on a cycle. Added feed once in the morning and again in the evening. Food and tap water were provided *ad libitum*. Ventilation once every 2 hours in the day and once every 4 hours in the night. After 3 days of acclimation, mice were labeled, weighed and grouped randomly.

Assay of SOD activity，MDA content and GSH-PX enzyme activity in serum and brain homogenate

The cerebellum was washed with 0.9% physiological saline repeatedly and removed fat and connective tissue after the anotomy of mice. The cerebellum was weighed after being filtered the surface water, homogenated in the tissue homogenisator. The homogenate was centrifuged in low temperature centrifuge 3,000 rpm for 15 min. The centrifugal supernate was stored in 4°C for 12 hours for later use.

Eyes in mice was picked after 5 weeks and blood was collected in 1.5 mL centrifuge tubes. The tubes were placed in centrifuge tube rack at room temperature for 2 hours. The blood clot and the edge of the tube were separated with needle. The tubes were stored in refrigerator at 4°C overnight for precipitation. The serum was transferred to centrifuge tubes with pipette and stored in low temperature.

The samples were assayed and the activity was calculated according to the assay method of the SOD, MDA, GSH-PX diagnostic kit. SOD, MDA, GSH-PX enzyme activity detection were performed under UV wavelength 550, 532, 412 nm respectively. Data obtained were analyzed by SPSS 11.5 for windows statistical software. The mean comparison between groups was analyzed by one-way analysis of variance. Pair-wise comparison in the group was analyzed by LSD method.

Validation of aging mice model

After 3 days of acclimation, a total of 36 or 8 week-old mice (18 ± 2 g) were grouped of both sexes were divided randomly to 3 groups with 12 mice in each group as follows. (1) blank control group: Mice were intraperitoneally injected with normal saline every day. (2) aging model group: Mice were injected with 150 mg/kg/day D-galactose aqueoaus solution at the dose of 10 mL/kg for two consecutive weeks, then were intraperitoneally injected with normal saline for three consecutive weeks. (3) positive drug group: Mice were injected with 150 mg/kg/day D-galactose aqueoaus solution at the dose of 10 mL/kg for two consecutive weeks, then were intragastricly administrated with Vitamin E at the dose of 180 g/kg for three consecutive weeks. The samples were obtained and SOD activity, MDA content and GSH-PX enzyme activity in serum and homogenate were assayed as described above.

Week-age choosing of experimental animals.

After 3 days of acclimation, 6 week-old mice (20 ± 2 g), 10 week-old mice (25 ± 2 g), 14 week-old mice (30 ± 2 g) of both sexes were grouped randomly as follows. (1) control groups included 6 week-old mice control group, 10 week-old mice control group, 14 week-old mice control group with 10 mice of both sexes in each group, mice in control groups were intraperitoneally injected with 150 mg/kg/day D-galactose aqueoaus solution at the dose of 10 mL/kg for three consecutive weeks, then mice were intragastric administrated with 3.6 mg/mL vitamin E dose at the dose of 10 mL/kg for three consecutive weeks respectively. (2) blank groups included 6 week-old mice blank group, 10 week-old mice blank group, 14 week-old mice blank group with 10 mice of both sexes in each group, mice in blank control groups were intrapetitoneally injected with normal saline at the dose of 10 mL/kg every day.

Establishment of dose-response linear relationship

According to the experimental results of mice week-age choosing, 10 week-old mice were used as the experimental animals in the experiment of the amount reaction of parallel line. A total of 140, 10 week-old mice (25 ± 2 g) were divided into 14 groups with 10 mice of both sexes in each group randomly. (1) anti-aging effect test of aging model mice: One group as blank control group was intraperitoneally injected with normal saline, the rest 6 groups were intrapepritoneally injected with 150 mg/kg/day D-galactose aqueous solution for 3 weeks, then were treated with 3.6 mg/mL Vitamin E by using intragastric administration at the doses of 11.20, 14.00, 17.4, 21.84, 27.32, 34.16 mg·kg-1 for 2 weeks respectively. (2) anti-aging effect test of normal mice: One group as blank control group were intraperitoneally injected with normal saline every day, the rest 6 groups were treated with 3.6 mg/mL Vitamin E by using intragastric administration at the doses of 11.20, 14.00, 17.4, 21.84, 27.32, 34.16 mg·kg-1 for 2 weeks respectively.

Anti-aging activity test of Radix polygoni multiflori preparata

After 3 days of acclimation, a total of 60, 10 week-age mice (25 ± 2 g) were divided into 6 groups with 10 mice of both sexes in each group randomly. (1) 3 groups of normal mice were included in control groups. All mice were intrapepritoneally injected with 150 mg/kg/d D-galactose aqueous solution for 3 weeks, then were treated with 0.7, 3.5, 17.5 mg/mL (concentration ratio 1:5:25) of Vitamin E by using intragastric administration at the dose of 20 mL/kg for 2 weeks respectively. (2) 2 groups of normal mice were included in positive groups. Each mice was intrapepritoneally injected with 150 mg/kg/day D-galactose aqueous solution for 3 weeks, then mice in positive groups were treated with the 0.8, 0.4 g/mL (concentration ratio was 1:2) decoction of Radix polygoni multiflori preparata at the dose of 20 mL/kg by using intragastric administration respectively. (3) 1 group was included in blank group. Mice in blank group were intrapetitoneally injected with equal amount of normal saline everyday..

Results

Radix polygoni multiflori preparata purchased met the standards recorded in the Chinese pharmacopoeia, and the inspection results were shown in Table I. Linearity was examined with selected concentration range with 6

Table I

Inspection results of Radix polygoni multiflori preparata

Test item	Inspection result	Requirements in Chinese Pharmacopoeia 2010 Version
Water	10.9%	< 12.0%
Total ash	7.3%	< 9.0%
Alcohol extract	6.5%	> 5.0%
Stibene glucoside	1.1%	> 0.70%
Free anthraquinone	0.2%	> 0.10%

levels and satisfactory linearity was obtained in the content determination of polysaccharide. The calibration curve was constructed by absorbance of the analytes versus the concentration (mg/mL). The linear regression equation and correlation coefficient (R2) was y = 13.443x—0.0147 (R2 = 0.9992, n = 6). Recovery study was conducted on a sample spiked with about 100% of known amount in the sample with 6 replicated analyses. The spiked samples were extracted and the amounts of the analyzes were quantified. The results showed that the average recovery was estimated to be 98.7% and relative standard deviation (RSD) was 3.0%. Method repeatability was evaluated by six replicated analyses of test solution. The RSD of the content of polysaccharide was 2.9%. Method precision was investigated by repeatedly analyzing the same set of sample solution and the RSD was 2.1%. The results showed that the polysaccharide content in the 0.4, 0.8 g/mL decoction were 80.1, 160.2 mg/mL respectively.

The HPLC results in the content determination of stibene glucoside showed that the stibene glucoside content in the 0.4 and 0.8 g/mL decoction were 404 and 808 mg/mL respectively. The HPLC results in the content determination of emodin and emodin monomethyl ether showed that the emodin and emodin monomethyl ether content in the 0.4 mg/mL decoction were 0.268, 0.176 mg/mL respectively, the emodin and emodin monomethyl ether content in the 0.8 g/mL decoction were 0.536, 0.352 mg/mL respectively.

Experimental results showed that 10 week-old mice were the most sensitive to SOD activity in homogenate (Figure 1). To sum up, 10 week-age mice were selected

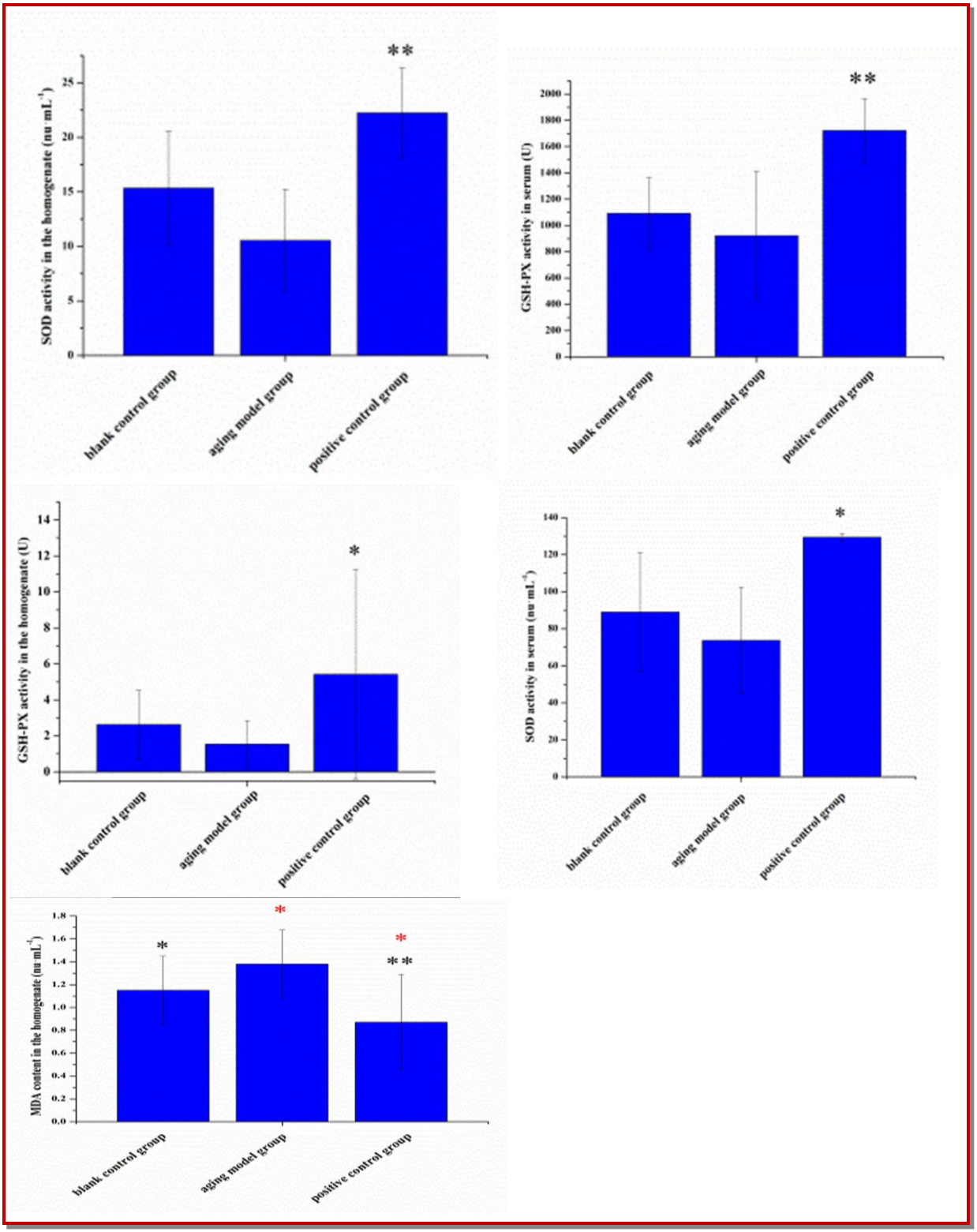

Figure 1: The changes of aging-associated markers. Stastically significant difference, *P<0.05 versus aging model group, **P<0.01 versus aging model group, *P<0.05 versus blank control group

Table II

Experimental results of the determination of parallel of amount reaction

Dose (mg/mL)	Control groups			Positive groups		Blank group
	0.7	3.5	17.5	400	800	
Total SOD activity (nU/mL)	14.9 ± 3.7	21.2 ± 4.2	26.0 ± 3.8	20.0 ± 4.5	21.6 ± 5.0	10.8 ± 3.3

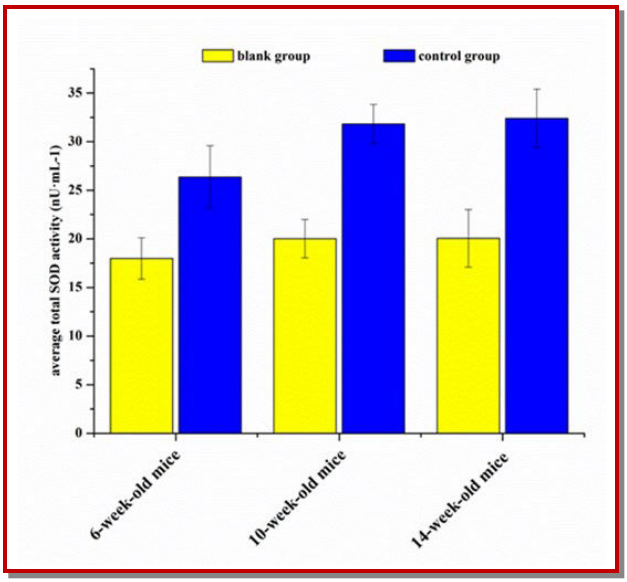

Figure 2: Week-age choosing of experimental animals

as the experimental animals.

The drug efficacy on creatures was taken as the response indicator in bioassay method, so dosage and response was the foundation of the bioassay method. The SOD activity in homogenate was used as the pharmacological indicator. The relationship between dosage and response was curve, so a variety of coordinate transformation methods were used to make the relationship between dosage and response represent linear relationship. The results of dose-response and the transformation (l g means base 10 logarithm) were showed in Figure 2. Experimental results showed that the data comparison between aging model mice and normal mice, the closer to 1 was the correlation coefficient, the result was closer to the real value as the establishment of the testing method. The linear relationship between the logarithmic dose and response in aging model mice was used in the research.

The results of amount reaction of parallel determination were showed in Table II. SPSS11.5 for window was used for data process and the data processing result was showed in Table III. Reliability test showed that the differences between doses and the differences in regression were very significant, the differences in deviations from parallel and the differences in deviations from the straight line were significant. Because S、T the two straight lines in were two parallel straight lines in the research, The potency and confidence interval were calculated according to the principle of parallel lines. The experimental results showed that the potency of Radix polygoni multiflori preparata was 0.573 times than that of vitamin E, the anti-aging effect of 1U vitamin E was equal to 0.04 g Radix polygoni multiflori, and the confidence limit was 15.7%.

SOD activity is reduced following age, so it is an important indicator of anti-aging medicine. GSH-PX is one of the anti-oxidant enzymes in the human body, and GSH-PX determination could be used as a key indicator of anti-oxidative capability. The determination results were showed in Figure 3. Also, the aging model copy were successful. SOD activity determination in the homogenate was the most sensitive and had better reproducibility, and the sample was easy to be prepared according to the results, so the SOD activity was used in the experiment.

Discussion

Free radical oxidation and anti-oxidant defense function stay in dynamic equilibrium in normal body.

Table III

Variance analysis and reliability testing results

Sources of variation	Freedom	Sum of squares of deviations	Variance	*F*	*Significance*
Test samples	1	0.1	0.1	< 1	
Regression	1	595.8	595.8	13.0	‡
Deviation from parallel	1	2.2	2.2	< 1	†
Deviation from the straight line	1	2.7	2.7	< 1	†
Between doses	4	600.9	155.2	3.4	‡
Errors	38	1742.1	45.8 (S^2)		
Total	46	2343.0			

‡ Very Significantly different between model groups and control groups at p<0.01; † Singnificantly different between model groups and control groups at p<0.05

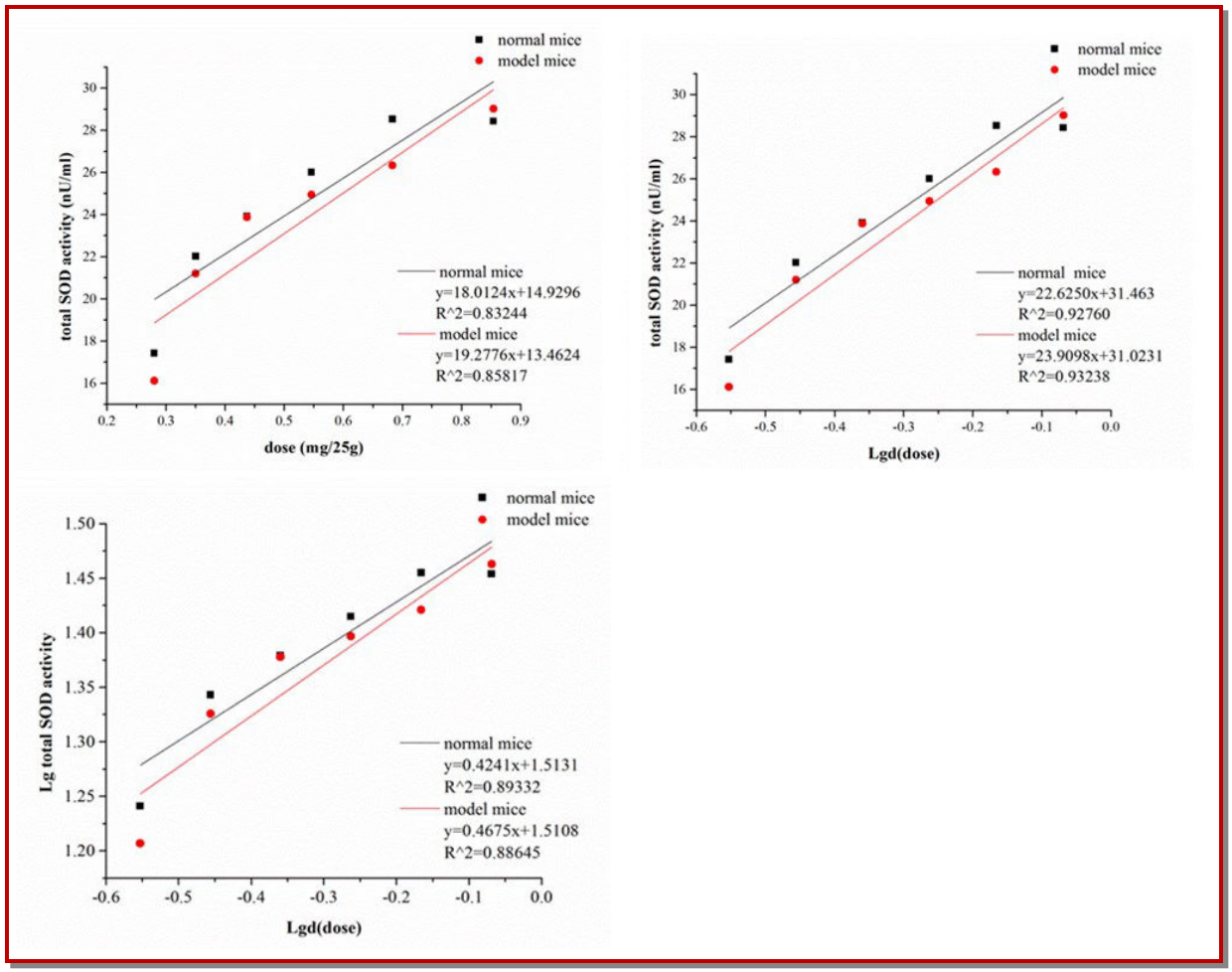

Figure 3: Dose and response linear relationship between normal mice and model mice

When the free radical scavenging enzyme and the defense function of non-enzymatic system were fade, this kind of balance was disordered, leading to body aging, such as metabolic disorders, immune organ degeneration and excess free radicals. D-galactose is a sugar that at higher levels converts to aldose and hydroperoxide during the catalysis of galactose oxidase, culminated in the generation of free radical. These modifications are substantially similar to the normal aging process demonstrated as neurological deterioration, diminished activity of anti-oxidant enzymes, and miserable immune responses.

(Mohammadirad et al, 2013). The Typical D-gal-induced aging mice model is the best one that meets clinical studies and the classic model used at home and abroad in recent years (Sun et al., 2008).

Recent research literature showed that anti-aging research was mostly used in the study on nourishing liver and kidney effect of RPMP in modern medicine study. Vitamin E is commonly supposed to have anti-aging effect, and was used as the positive substances. MDA was peroxide products after polyunsaturated fatty acids being exposed by ROS. MDA content could reflect ROS content to infer the ROS damage to the body indirectly, as a reliable and clear sign of cell or organ aging. Superoxide anion, the main free radicals in the organism, is harmful to body in most cases and is one main cause leading to aging.

Biological potency assessment is to compare the specific reaction produced by test articles and equal reference substance, through the proportional calculations between equivalent reaction dose to measure the potency of test solution, that is, comparison verification. The common experimental designs in bioassay method are direct measurement method, parallel line assay method, slope ratio method and average dosage comparison method. Parallel line assay method is the most common and popular, it can be divided into amount reaction parallel line model and quality reaction parallel line model depending on dose-response. The research topic was mainly to study on amount reaction, so amount reaction of parallel line determination was used in the experiment.

The arithmetric of amount reaction of parallel line determination could be divided into basic arithmetic and simple arithmetic. Simple arithmetic formula is brief, works fast and has been adopted by Chinese pharmacopoeia. But in order to build the biological control method system of the quality of TCM, the ideas of the potency test of biological products should not be benefited from, but also the arithmetic suitable for the character of TCM should be considered. Due to that the quality of TCM is affected by many links and many ways, sample totality fluctuation ranges huge, so basic arithmetic is more suitable for bioassay data processing of TCM. Basic arithmetric pair (3·2) was used in the research, so 3 doses of Vitamin E was used in control groups and 2 doses of decoction in positive groups in the research. As the relationship between l g (dose) and response was linear relationship, the biological potency could be calculated according to amount reaction. The calculation principle includes lines paralization and the reliability test.

Conclusion

Bioassay method of anti-agingeffect of Radix polygoni multiflori preparata was established preliminarily, and it can be used for its quality control.

Ethical Issue

The protocol of the study was approved by the Ethics Committee of Guangdong Pharmaceutical University, China. The investigation was conducted in accordance with the ethical principles of animal use and care.

Conflict of Interest

The authors declare no conflicts of interest.

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