Protective Effects of Loofah Leaf-extract on Nerve Cell Injury

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ABSTRACT

Alzheimer's disease (AD) is a debilitating neurodegenerative disorder characterized by increased β-amyloid (Aβ) deposition and neuronal dysfunction leading to impaired learning and recall. Ageing and induced oxidative stress are among proposed risk factors. We studied the protective effect of Loofah leaf-extract (LL) on the injury of PC12 cells induced by amyloid β protein ($A\beta_{25-35}$). Neurons injury model was induced by $A\beta_{25-35}$. MTT assay was used to detect the cellular viability after different concentrations of Loofah leaf-extract. The activity of superoxide dismutase (SOD), malondialdehyde (MDA) and glutathione peroxidase (GSH-Px) were detected. The expression of Bcl-2 and Bax were detected by Western Blotting. Results: Compared with the control group, the cellular viability of Loofah leaf-extract group is increased, the activity of GSH-Px and SOD are significantly increased ($P<0.01$, $P<0.05$), MDA content decreased significantly, the expression of Bax is decreased and Bcl-2 is increased ($P<0.05$, $P<0.01$). These results indicate that Loofah leaf-extract can protect PC12 cells damage.

Key words: Alzheimer's Disease, Loofah Leaf-extract, PC12 Cells, $A\beta_{25-35}$

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Introduction

Luffa, a plant of *Cucurbitaceae*, Loofah leaves can be used as medicine (Su *et al*., 2014), been proved in recent years to have such physiological functions as enhancing immunity, anti-stress, detoxification, hemostasis, and promoting growth, Modern pharmacological studies found that loofah leaves contain a terpenoid saponin substance called L-6a, which can increase blood supply to the brain, protect brain cells, and promote the release of somatostatin in the brain neuron, and prevent Alzheimer's disease (Qi *et al*., 1999; Li *et al*., 2001). The present study aims to investigate the neuroprotective effect of Loofah leaf-extract on PC12 cells injury induced by β-amyloid protein ($A\beta_{25-35}$)(Zeng *et al*., 2004; Choi *et al*., 2010), and to explore the neuroprotective mechanism of Loofah leaf-extract on AD (Ghofrani *et al*., 2015).

Methods

Drugs and reagents

The Loofah leaf-extract was provided by Xi'an SR Bio-Engineering Co, Ltd. The PC12 cells were purchased from Beijing BeNa Chuanglian Biotechnology Research Institute, MTT and $A\beta_{25-35}$ from Sigma, MDA, SOD and GSH-Px detection kit from Nanjing Jiancheng Bioengineering Institute, DMEM medium, fetal bovine serum, and horse serum from Hangzhou Sijiqing Engineering Materials Research Institute, and Bcl-2 and Bax antibodies were purchased from Santa Cruz Biotechnology. All other reagents are analytical reagents produced in China.

Instruments

Carbon dioxide incubator (Thermo), EPOCH microplate reader (BioTek Instruments, Inc), high speed freezing centrifuge (Shanghai Canspec

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Scientific & Technology Co, Ltd.), Electrothermal constant temperature water tank (Ningbo Tianheng Instrument Factory), superclean bench (TBD Biological Instrument Factory), gel scanning imaging analysis system (Startganee of USA).

Culture of PC12 cells and establishment of injury model

The cells were cultured in CO_2 incubator of 37°C and 5% CO_2 saturation humidity, with high glucose DMEM medium containing 15% fetal bovine serum, 100U/ml penicillin and 100 U/ml streptomycin. The liquid was changed once in 2~3 days. The adherent cells were digested with 0.25% trypsin, and the number of the cells were adjusted to 1×10^5 cells/ml. Then they were inoculated into 96-well cell culture plates and cultured into logarithmic growth phase cells for various indicators testing. PC12 cells cultured in vitro were divided into 6 groups: blank group, model group, group with high-dose of Loofah leaf-extract, group with medium dose of Loofah leaf-extract, group with low-dose of Loofah leaf-extract. The model group was incubated with $A\beta_{25-35}$ after aging treatment (final concentration of 20umol/L) for 36 hours, to establish AD cell model. The groups with various doses of Loofah leaf-extract were incubated overnight at final concentrations of 75μg/ml, 50μg/ml and 25μg/ml for 2 hours, then all incubated at a final concentration of 20umol/L$A\beta_{25-35}$ for 36 hours. Each group had 8 wells, each with 200ul.

Detection of cell viability by MTT colorimetric assay

The PC12 cells in logarithmic growth phase were prepared into a suspension of 2×10^5 cells/ml, which were then placed in a 96-well culture plate for plating, 200ul for each well. After the action with different processing factors for 24 hours, they were inspected by MTT assay with supernatant being discarded. Then add 180μl of culture medium, and 20μl MTT to each well. After being cultured at 37°C for 4 hours, the culture medium was aspirated, and 150μl of DMSO was added. After shaking for 10 minutes, the absorbance of the specimen was read at a wavelength of 570nm on a microplate reader. The ratio of the absorbance of each group to that of the control group was taken as the viability of the cells. Then calculate the following formula according to the relative activity of cells (Yu *et al*., 2012; Zhou *et al*., 2012): relative activity=mean absorbance of treated group/mean absorbance of the blank control group×100%.

Effects of Loofah leaf-extract on antioxidant capacity of PC12 cells induced by oxidative stress

Select the cells in the logarithmic growth period, for the preparation of suspension of 2×10^5 cell/ml with trypsin cells, which was then inoculated into the 6-cell culture plate. After being pretreated with final concentrations (25μg/ml, 50μg/ml, and 75μg/ml) for 30 minutes, the Loofah leaf-extract was then incubated with final concentration of 20umol/$A\beta_{25-35}$ for 24 hours. After completion of administration and $A\beta_{25-35}$ treatment, the collected cells were ultrasonicated and centrifuged at 4000r/min at 4°C for 10 minutes. The supernatant was collected and the activity of SOD, MDA and GSH-Px in cell homogenate was tested by kit method.

Western blotting analysis

After 24-hour drug treatment, the cells were harvested and washed. The total protein was separated by SDS-PAGE electrophoresis. Then the protein was transferred to PVDF membrane, which was sealed with 5% skim milk powder for 2 hours at room temperature. After being washed 3 times with TBST, the sealed membranes were placed in hybridization bags. Rat Bcl-2, Bax and beta-actin- were incubated overnight at 4°C, and then washed 3 times with TBST. Horseradish peroxidase-labelled rabbit anti rat IgG was incubated at 37°C for 1 hour, washed with TBS and developed by ECL method. Then the gray value of each band was analyzed by gel imaging system and the gray ratio of the target band to the internal reference calculated.

Statistical analysis

The experimental data were expressed as X±S. SPSS16.0 software was used for statistical analysis, and t test to compare the differences between groups. If $P<0.05$, then there considered to be significant differences between groups.

Results

Protective effect of Loofah leaf-extract on PC12 cells induced by $A\beta_{25-35}$

Compared with the blank group, the viability of the cells treated with 20umol/L of $A\beta_{25-35}$ is significantly lower, which is only 59.20%. Thus there is significant difference ($P<0.01$). When PC12 cells are pretreated with Loofah leaf-extract and then treated with $A\beta_{25-35}$, the viability of PC12 cells is significantly increased, and shows a trend of dose-dependent tolerance. The cellular viability reaches 76.11%, 69.27% and 61.12% respectively

after treatment with Loofah leaf-extract (75μg/ml, 50μg/ml, and 25μg/ml). The results are as shown in Figure 1.

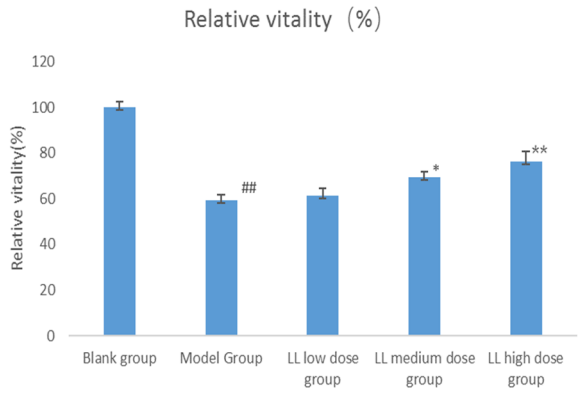

Figure 1. MTT Testing of protective effects of different concentrations of Loofah leaf-extract on cytotoxicity of PC12 cells induced by $A\beta_{25\text{-}35}$

*Notes: when compared with the blank group, ##P <0.01; when compared with the model group, *P <0.05, **P <0.01*

The effect of Loofah leaf extract on MDA content in $A\beta_{25\text{-}35}$ induced PC12 cells

Compared with the blank group, the content of MDA in PC12 cells treated with 20umol/L of $A\beta_{25\text{-}35}$ is significantly increased (P<0.01). Compared with model group, groups with different doses of Loofah leaf-extract can significantly decrease MDA content (P<0.01, 0.05). with a certain dose correlation, as shown in Figure 2.

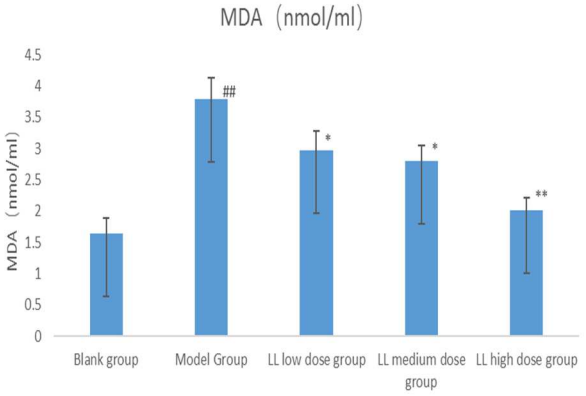

Figure 2. Effect of Loofah leaf-extract on, MDA of PC12 cells induced by $A\beta_{25\text{-}35}$

*Notes: when compared with the blank group, #P <0.05, ##P < 0.01; when compared with the model group, *P <0.05, **P < 0.01.*

Effect of Loofah leaf-extract on the activity of SOD in PC12 cells induced by $A\beta_{25\text{-}35}$

Compared with the blank group, the activity of SOD in PC12 cells treated with 20umol/L of $A\beta_{25\text{-}35}$ is significantly decreased (P<0.01). Compared with model group, groups with different doses of Loofahleaf-extract can significantly raise SOD activity (P <0.01, 0.05), as shown in Figure 3.

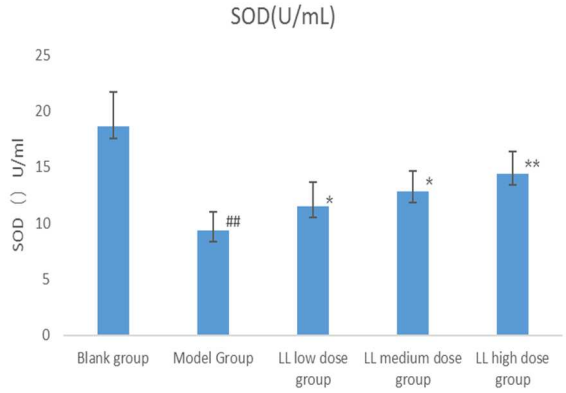

Figure 3. Effect of Loofah leaf-extract on, SOD of PC12 cells induced by $A\beta_{25\text{-}35}$

*Notes: when compared with the blank group, #P <0.05, ##P < 0.01; when compared with the model group, *P <0.05, **P < 0.01.*

Effect of Loofah leaf-extract on the activity of GSH-PX in PC12 cells induced by $A\beta_{25\text{-}35}$

Compared with the blank group, the activity of GSH-PX in model group is significantly decreased (P<0.01). Compared with model group, groups with different doses of Loofah leaf-extract can significantly raise GSU-PX activity (P <0.01, 0.05), as shown in Figure 4.

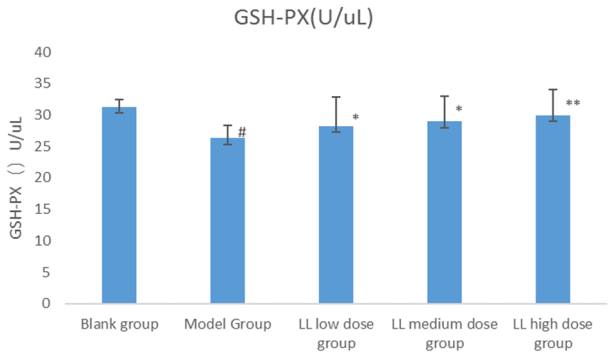

Figure 4. Effect of Loofah leaf-extract on, GSH-PX of PC12 cells induced by Aβ25-35

*Notes: when compared with the blank group, #P <0.05, ##P < 0.01; when compared with the model group, *P <0.05, **P < 0.01.*

Effect of Loofah leaf-extract on the expression changes of Bcl-2 and Bax in PC12 cells Induced by $A\beta_{25-35}$

Compared with the blank group, in the PC12 cells treated with 20umol/L of $A\beta_{25-35}$, Bax expression is significantly raised, Bcl-2 expression is significantly decreased (P<0.01), and Bcl-2/Bax expression ratio declines. Compared with the model group, in the groups with high, medium and low doses of Loofah leaf-extract, Bax expression is significantly decreased (P<0.01, 0.05), and Bcl-2 expression ratio is significantly raised (P<0.01, 0.05), The ratio of Bcl-2/Bax expression is significantly raised compared with model group, as shown in Figure 5 and Figure 6.

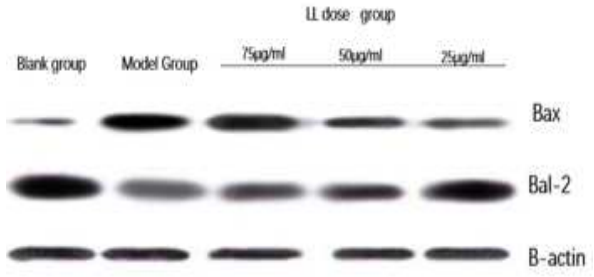

Figure 5. Effect of Loofah leaf-extract on protein expression induced by $A\beta_{25-35}$

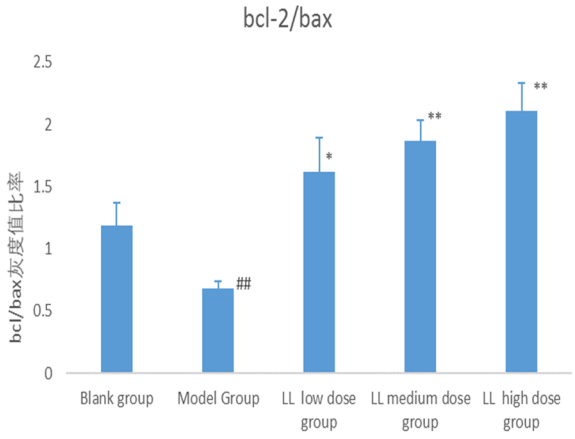

Figure 6. Effect of Loofah leaf-extract on expression changes of Bcl-2/Bax in PC12 cells induced by $A\beta_{25-35}$
*Notes: when compared with the blank group, ##P <0.01; when compared with the model group, *P <0.05, **P <0.01.*

Conclusions

The pathological feature of Alzheimer's disease (AD) is senile plaque (SP) (Ong *et al*., 2015), whose core is β-amyloid (Aβ). In normal physiological conditions, Aβ has the function of nutritional factor, but high concentration of long chain Aβ tends to be neurotoxic (Bagheri *et al*., 2011; Zhu *et al*., 2009). Aβ production and increased accumulation is the main mechanism for causing neuronal degeneration and death. Aβ can produce peroxide and free radicals through a variety of ways, thus aggravating the damage of oxidative injury to nerve cells (Liu *et al*., 2015).

Recent studies have shown that the degeneration of human body with age is the result of the side effects of excessive free radicals produced during normal metabolism of cells. MDA is a product of lipid peroxidation.If the body's SOD, GSH-PX and other antioxidant enzyme decrease, the free radical formation in the body will decline, the peroxidation will be strengthened, and the tissue and the cell will be destroyed (Li *et al*., 2014; Andersen *et al*., 2003), so that the function of the tissue will decline and organism age (Khan *et al*., 2012). This study shows that $A\beta_{25-35}$ can lead to MDA level increase and SOD, GSH-PX level decrease in PC12 cells. However, the PC12 cells pretreated with Loofah leaf-extract significantly increases SOD, GSH-PX and decrease MDA in cells induced by $A\beta_{25-35}$, thereby reducing the level of oxidative stress within the cell. Reducing or blocking the action of lipid peroxides can protect the body from the damage of hydrogen peroxide and delay the injury of cell senescence, thereby preventing the diseases caused by free radicals.

The balance of anti-apoptotic gene Bcl-2 and pro-apoptotic gene Bax in Bcl family plays an important role in deciding whether or not the cells enter the apoptosis pathway, which means, the ratio of Bc1-2/Bax determines whether or not apoptosis occurs (Wu and Li 2010). The experimental results show that the expression of Bc1-2 protein is decreased, the expression of Bax increased and the ratio of Bc1-2/Bax decreased in the model group. Thus the apoptosis of cells is exacerbated. After the intervention with Loofah leaf-extract, Bcl-2 expression is increased, Bax expression decreased, and the ratio of Bcl-2/Bax increased, thus apoptosis is inhibited. It indicates that Loofah leaf-extract can inhibit cell apoptosis. The protective effect of Loofah leaf-extract on brain cells of mice with AD may be achieved by up-regulating Bcl-2 protein, down-regulating the expression of Bax protein and inhibiting apoptosis, thereby relieving Alzheimer's disease (Goodsell 2002; Paul-Samojedny *et al*., 2005).

In conclusion, Loofah leaf-extract has obvious protective effect on PC12 cell injury induced by $A\beta_{25-35}$, which may be realized by enhancing the anti-oxidative stress and anti-apoptosis effect of PC12 cells, but its mechanism of AD prevention and treatment remains to be further studied.

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