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Preliminary screening of active components in regulating autophagy in *Ziziphora clinopodioide* Lam.

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Abstract: *Ziziphora clinopodioide* Lam, a traditional Chinese medicinal plant, has been used to treat hypertension, coronary heart disease and other cardiovascular diseases, autophagy plays an important role in these diseases. This study investigated the effects of *Z. clinopodioides* and its active components on autophagy using cell biology. Normal rat kidney (NRK) cells transfected with green fluorescent protein-associated microtubule-protein 1 light Chain 3 (GFP-LC3) were intervened with different doses of ethanol and water extracts of *Z. clinopodioide*s and the active components of *Z. clinopodioide*s. After 4 hours treatment, the autophagy spot aggregation in NRK cells was photographed and observed by laser scanning confocal microscopy. The results showed that the water and ethanol extracts of *Z.* clinopodioides can activate autophagy, the effect of activating autophagy was more significant, when the dose was increased. Five components including chrysin, luteolin, quercetin, oleanolic acid and ursolic acid were identified as the active principles in activating autophagy. This research may provide a reference for the further study of mechanism and material basis of *Z. clinopodioides* in treatment of cardiovascular diseases.

Keywords: *Ziziphora clinopodioides* Lam., autophagy, screening.

INTRODUCTION

Ziziphora clinopodioides Lam, a traditional Chinese medicinal plant, is widely distributed in China, Mongolia, Russia, Iran, Turkey, Kazakhstan and Kyrgyzstan. People use it for the treating heart disease, hypertension, asthma, sweating, palpitation, insomnia, oedema, cough, bronchitis, lung abscesses and other diseases (Tian *et al*., 2011). This plant has many biological activities, such as antibacterial, antimicrobial, anti-inflammatory and anti-oxidant properties; a relaxing effect on the vascular system; and an immunity-boosting effect on laying hens (Nobakht *et al*., 2011, Senejoux *et al*., 2012, Shahla *et al*., 2012). People mainly focused on its clinical application and chemical composition, including diosmin, linarin, hyperin, quercetin, chrysin, luteolin, rutin, baicalein, isoquercitrin, caffeic acid, rosmarinic acid, protocatechuic acid, oleanolic acid, ursolic acid and other ingredients (Tian *et al*., 2012, Smejkal *et al*., 2016); however, its mechanism and material basis in treating cardio-vascular diseases remain unclear.

Autophagy refers to "self-eating" where cells degrade their cellular constituents to maintain cellular homeostasis as a normal response to stresses, such as hypoxia, oxidative stress, starvation and toxic reactions (Mizushima *et al*., 2008). Some studies support that autophagy is involved in the occurrence and development of atherosclerosis (AS),which is the main pathological basis of cardiovascular and cerebrovascular diseases (Shao *et al*., 2016). Normal rat kidney (NRK) cells stably transfected with a fusion protein between green fluorescent protein and light chain 3 (GFP-LC3) were used to investigate autophagy (Mi *et al*., 2015). Drug screening with autophagy as the target has become a new research focus (Shu *et al.*, 2012). Therefore, this study investigates the preliminary screening of *Z. clinopodioides* and its main components in regulating autophagy through cell biology.

MATERIALS AND METHODS

Chemicals and reagent

A total of 0.25% trypsin, Foetal bovine serum, Dulbecco's phosphate-buffered saline (DPBS) and Dulbecco's modified Eagle medium (DMEM) were purchased from Hyclone (USA). Dimethyl sulfoxide (DMSO) was purchased from Sigma (USA). NRK with GFP-LC3 was given by Professor Mina from the Experimental Centre of Xinjiang Medical University (China). Diosmin, linarin, hyperin, rutin, quercetin and protocatechuic acid were purchased from China's food and drug inspection Institute. Chrysin, baicalein, luteolin, isoquercitrin, caffeic acid, rosmarinic acid, oleanolic acid and ursolic acid were purchased from Aladdin (China).

Plant material

Z.clinopodioides specimens were collected from Tuoli of Urumqi Region, Xinjiang Province, People's Republic of China in July 2016 and identified by Prof. Hai-yan Xu of Xinjiang Medical University. The specimen (NO. 20160708-03) is stored in the Traditional Chinese Medicine Ethnical Herbs Specimen Museum of Xinjiang Medical University.

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Preparation of alcohol extract and water extract of Z. clinopodioides Lam.

A total of 8 grams *Z. clinopodioides* Lam crude powder was weighted. A total of 200 mL 70% ethanol was added and extracted through heating reflux for 1.5h. The extraction process was repeated three times. All substances were filtered and evaporated to dryness under vacuum. The water extract of *Z. clinopodioides* Lam was obtained in the same way as the alcohol extract, but the solvent is water (Tian *et al.*, 2012). The residue was weighed and 1.2084g powder was obtained from the ethanol extract, while 1.5308 g powder was obtained from the water extract. Next, both powders were stored at -20^{o}C prior to further analysis.

Cell culture

The normal rat kidney (NRK) cells with GFP-LC3 was cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 µg/mL streptomycin maintained at 37 ^{o}C and normal 5% CO_2 culture medium. Cells were inoculated in the culture dish (20 mm diameter) at 70-80% confluence.

Effects of Z.clinopodioides and its components on NRK with GFP-LC3

NRK cells were seeded into a culture dish (20 mm) at a density of 1×10^{6}cells/well and cultured at 37^{o}C for 24h. NRK cells were pre-treated with water and ethanol extracts of *Z. clinopodioides* Lam. (1,2,4mg/mL) and their components (hyperin, rutin, quercetin, luteolin, caffeic acid, protocatechuic acid and isoquercitrin were 50 µmol/L; diosmin and baicalein were 30 µmol/L; linarin, chrysin, ursolic acid, oleanolic acid and rosmarinic acid/ were 20 µmol/L). The control group was only given DMEM. The starvation group was given Dulbecco's phosphate buffer saline. According to literature and pre-experiment, we chose 60% as the safe dose and screening standard with 4 hours treatment (Mi *et al.*, 2015). All drugs were dissolved in DMSO and diluted in DMEM (final concentration in cells of DMSO<0.1%), One milliliter in per culture dish. Autophagy spot aggregation in NRK cells with GFP-LC3 was pictured and observed by laser scanning confocal microscopy (NikonC2, Japan) under ×60 vision. We calculated the number of average autophagy in each cell body and found at least 50 cells in each sample.

STATISTICAL ANALYSIS

Statistical analysis was performed using SPSS 20.0 (Chicago, USA). Data were expressed as mean ± standard error of the mean for multiple comparisons. The data were analyzed by the one-way analysis of variance (ANOVA). $P<0.05$ was considered statistically significant. Experiments were performed in triplicates.

RESULTS

Effects of water and ethanolic extracts of Z. clinopodioides Lam on NRK cells with GFP-LC3

As shown in fig. 1, the water and alcohol extracts of *Z. clinopodioides* Lam can induce GFP-LC3 puncta, there are many GFP-LC3 puncta, just like the starvation group. The numbers of autophagy puncta significantly increased in a dose dependent manner. 4 mg/mL group of water and alcohol extracts of *Z. clinopodioides* Lam exhibited the most significant effect among the drug groups (fig. 1E.H). However, the control group showed few points of autophagy (fig.1A). Compared with the control group, the number of autophagy spot aggregation in the ethanol and water extracts of *Z. clinopodioides* was significantly increased, while there were no significant difference between the group of water and alcohol extracts of *Z. clinopodioides* Lam (4mg/mL) and the starvation group (fig.1 I).

Effects of active components of Z. clinopodioides Lam on NRK cells with GFP-LC3

As shown in fig. 2, after intervention with different components of *Z. clinopodioides* Lam. on NRK cells with GFP-LC3, five of these components, including chrysin, luteolin, quercetin, oleanolic acid and ursolic acid, can significantly activate autophagy. There are many GFP-LC3 puncta, just like the starvation group. Ursolic acid is the most significant in activating autophagy (fig. 2N). However, the others cannot activate autophagy there are few GFP-LC3 puncta (fig. 2). Compared with the control group, the number of autophagy spot aggregation in the components of Z. clinopodioides (chrysin, luteolin, quercetin, oleanolic acid and ursolic acid) was significantly increased, while other components did not. Compared with the starvation group, ursolic acid group had the largest number of autophagy spot aggregation and quercetin group had the same number of autophagy spot aggregation (fig. 2Q).

DISCUSSION

Atherosclerosis (AS) is pathological basis of cardiovascular and cerebrovascular diseases, such as coronary heart disease, myocardial infarction and stroke. AS is treated mainly using statins, antiplatelet therapy and interventional therapy. However, even under statin therapy, the residual risk of cardiovascular events remains high at approximately 22%. Moreover, statins have serious adverse reactions (such as myositis, myalgia and rhabdomyolysis). All these reduce the drug tolerance and compliance in patients with long-term use (Zhang, 2012; Saito, 2007). Therefore, developing new therapeutic drugs in AS is needed. The few side effects of natural drugs have generated research interest.

Ziziphora clinopodioide Lam, a traditional Chinese medicinal plant, has been used to treat hypertension, coronary heart disease and other cardiovascular diseases for a long period. Much of the biological activity of the plant has been reported (Nobakht *et al*., 2011, Shahla *et al*., 2012), such as its anti-bacterial, antimicrobial, anti-inflammatory and antioxidant properties, but its material basis and mechanism in treating cardiovascular diseases remain unclear.

Autophagy plays an important role in regulating AS formation and the stability of atherosclerotic plaques (Schrijvers *et al*., 2011; Liu *et al*., 2015). Physiological autophagy is a self-protective mechanism of cells and is beneficial to cell growth and development, which are important in protecting cells against metabolic stress and oxidative damage, synthesis, degradation and recycling of cellular homeostasis and cell product recycling. However, excessive autophagy may lead to metabolic stress, degradation of cellular components and cell death. Autophagy is suggested as a protective mechanism in AS development, which is caused by oxidative modification

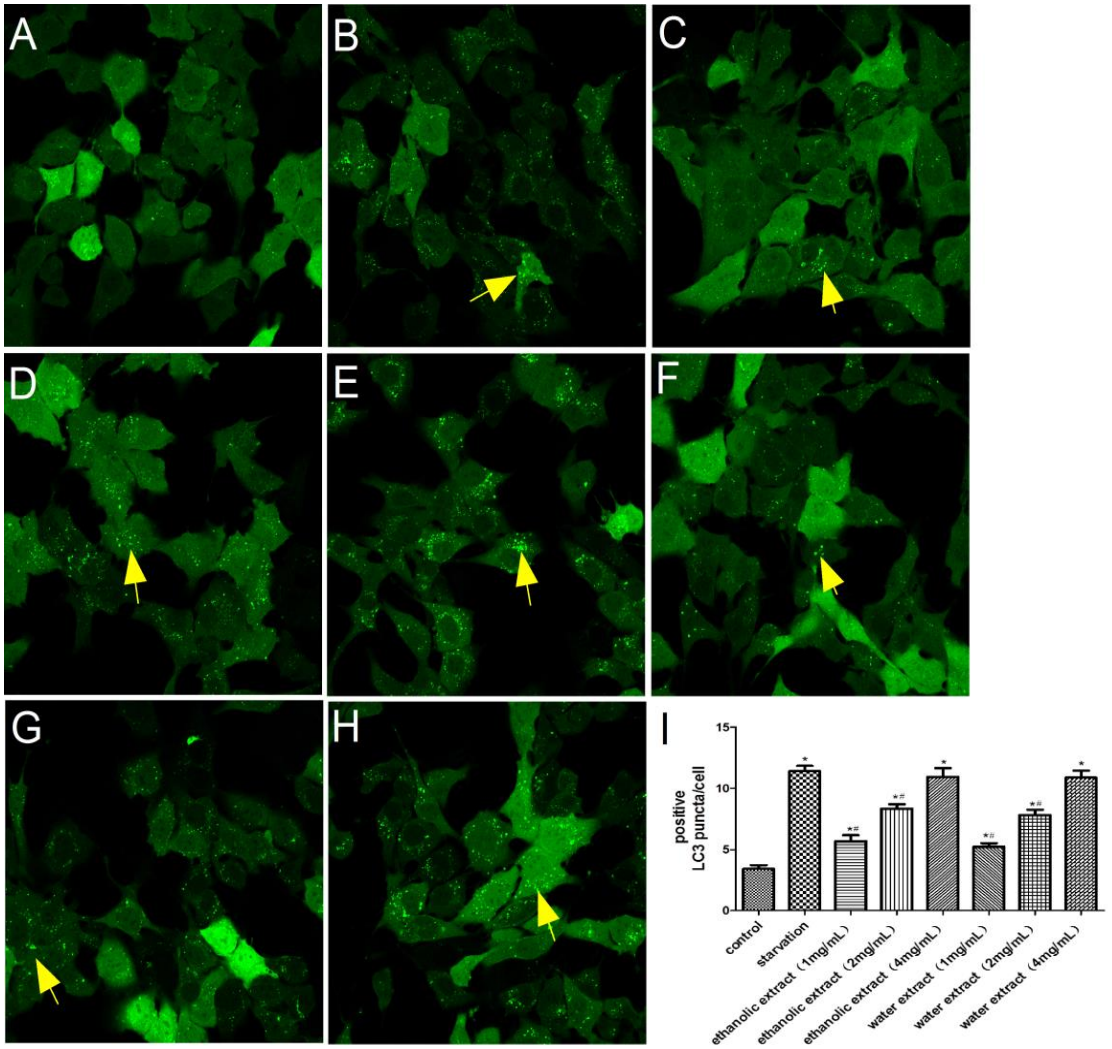

Fig. 1: Effects of different concentrations of water and ethanol extracts of *Z. clinopodioides* on NRK cells with GFP-LC3.NRK cells were pretreated with different concentrations of water and ethanol extracts *Z.clinopodioides* or DPBS for 4h.Autophagy spot aggregation in NRK cells with GFP-LC3 was pictured by laser scanning confocal microscopy, and calculated the number of average autophagy in each cell body.A: The control group. B: The starvation group. C: Alcohol extract of *Z.clinopodioides* (1mg/mL). D: Alcohol extract of *Z.clinopodioides* (2mg/mL). E: Alcohol extract of *Z.clinopodioides* (4mg/mL). F: Water extract of *Z.clinopodioides* (1mg/mL). G: Water extract of *Z.clinopodioides* (2mg/mL).H: Water extract of *Z.clinopodioides* (4mg/mL). I: The bar graph represents the statistical results of the positive LC3 puncta in per cell. The yellow arrow showed autophagy puncta. The positive LC3 puncta was described with mean ± SD (n=3). Data are representative of three independent experiments.*P<0.01 vs control group; $^{\#}$P<0.01 vs starvation group).

of proteins; moreover, autophagy defects aggravate AS (Menghini *et al*., 2014). Screening for drugs with autophagy as a target has recently become an interesting topic. Research data showed that the effective ingredients of Chinese medicine, such as curcumin and resveratrol, can induce moderate autophagy to protect from vascular endothelial cell injury (Chen *et al*., 2013; Han *et al*., 2012). Mammalian LC3 is an analogue of the yeast autophagy-related gene, *Atg*8, which undergoes ubiquitin-like post-translational modifications and is localized on the surface of the autophagic membrane. The exogenous LC3 molecular marker GFP was transfected into cells, and when cell autophagy occurs, GFP-LC3 will gather in the autophagic membrane; moreover, multiple green fluorescent spots were observed with the fluorescence microscope. Without autophagy, GFP-LC3 protein will be dispersed in the cytoplasm. Thus, we can determine whether autophagy occurs in the cells under fluorescence microscope through exogenous transfection (Ni *et al*., 2011). In the case of starvation, the cells will produce physiological autophagy and play a protective role.

This study revealed that the ethanol and water extracts of *Z. clinopodioides* Lam can activate autophagy. The active components including chrysin, luteolin, quercetin, oleanolic acid and ursolic acid showed significant effects

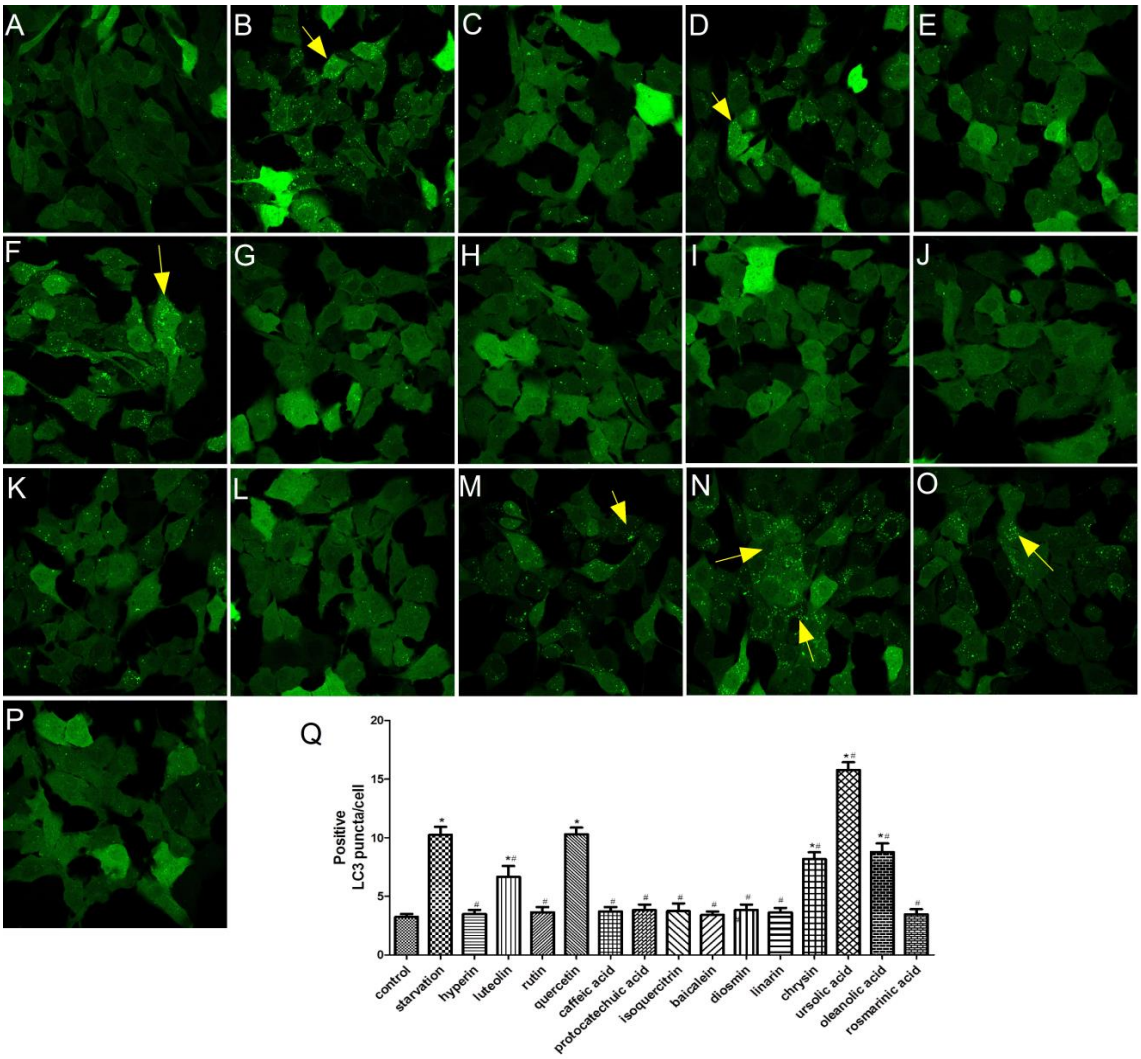

Fig. 2: Effects of components *of Z. clinopodioides* on NRK cells with GFP-LC3. NRK cells were pretreated with the components of *Z. clinopodioides* or DPBS for 4h.Autophagy spot aggregation in NRK cells with GFP -LC3 was pictured by laser scanning confocal microscopy, and calculated the number of average autophagy in each cell body. A: The control group. B: The starvation group. C: hyperin. D: luteolin. E: rutin. F: quercetin. G: caffeic acid. H: protocatechuic acid. I: isoquercitrin. J: baicalein. K: diosmin. L: linarin. M: chrysin. N: ursolic acid. O: oleanolic acid. P: rosmarinic acid. Q: The bar graph represents the statistical results of the positive LC3 puncta in per cell.The yellow arrow showed autophagy puncta. The positive LC3 puncta was described with mean ± SD (n=3). Data are representative of three independent experiments.*P<0.01 vs control group; $^{\#}$P<0.01 vs starvation group).

in activating autophagy. Ursolic acid is the most significant, which is consistent with research (Leng *et al*., 2016). The activation of autophagy in *Z. clinopodioides* Lam and its active ingredients may provide a possible strategy for AS prevention and treatment.

CONCLUSION

The ethanol and water extracts of *Z. clinopodioides* Lam can activate autophagy. *Z. clinopodioide* Lam can partly activate autophagy due to the five active ingredients, chrysin, luteolin, quercetin, oleanolic acid and ursolic acid. This paper may provide a reference for the further study of mechanism and material basis of *Z. clinopodioides* in treatment of cardiovascular diseases.

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