STACHYDRINE PROTECTS NEONATAL RATS FROM HYPOXIC-ISCHEMIC BRAIN INJURY BY REGULATING HDAC ACTIVITY, OXIDATIVE STRESS AND NEURONAL INFLAMMATORY RESPONSE

Shan Chen[1], Haiyang Lin[2*], Juan He[1], Ling Wang[1], Xiaowen Chen[1], Li Tao1, Heng Su[1]
[1]The Neonatal Intensive Care Unit, Guangzhou Women And Children's Medical Center, Guangzhou 510630, Guangdong Province, China - [2]The Pediatric Intensive Care Unit, Guangzhou Women And Children's Medical Center, Guangzhou 510630, Guangdong Province, China

ABSTRACT

Objective*: To explore the effect and mechanism of artesunate on pain associated with osteoarthritis (OA) and the inflammatory response of chondObjective: The objective was to study the protective effect of stachydrine on hypoxic-ischemic brain injury in neonatal rats by regulating HDAC activity, oxidative stress and neuronal inflammatory response.*

Methods*: Thirty-two newborn SD rats were divided into different groups, namely the control, model group, stachydrine 5mg/kg, and 10 mg/kg. In addition to the control group, the ischemic and hypoxic brain injury model was established by coronary artery ligation. The infarct area, apoptosis rate of brain tissue, HDAC activity, oxidative stress index, and inflammatory factor levels were observed.*

Results*: The area of cerebral infarction in the cortex of rats in 5 mg/kg and 10 mg/kg dose groups was significantly lower than that in the model group ($P < 0.05$). The apoptosis rate of brain tissue of stachydrine 5 mg/kg and 10 mg/kg groups was significantly lower than that of the model group ($P < 0.05$). HDAC activity in brain tissue of stachydrine 5 mg/kg and 10 mg/kg groups was significantly lower than that of the model group ($P < 0.05$). The expression of acetylated protein H3 and H4 in brain tissue of rats in stachydrine 5mg/kg and 10mg/kg groups were significantly higher than that in the model group ($P < 0.05$). The levels of SOD, GSH PX, and T-AOC in brain tissue of stachydrine 5 mg/kg and 10 mg/kg groups were significantly higher than those of the model group, and the MDA level was significantly lower than that of the model group ($P < 0.05$). The levels of TNF-α, IL-1β, ICAM-1, and VCAM-1 in the 5 mg/kg and 10 mg/kg dose groups were significantly lower than those in the model group ($P < 0.05$).*

Conclusion*: Stachydrine can improve the damage degree of neonatal rats with hypoxic-ischemic brain injury and has an obvious protective effect. Its mechanism may be achieved by inhibiting HDAC activity, regulating oxidative stress and neuronal inflammatory response.*

Keywords: *stachydrine, HDAC activity, oxidative stress, inflammatory response, hypoxic-ischemic brain injury.*

DOI: 10.19193/0393-6384_2022_3_245

Received *March 15, 2021;* ***Accepted*** *January 20, 2022*

Introduction

Hypoxic-ischemic brain injury is a brain lesion caused by cerebral ischemia and hypoxia and has various causes. It is a common clinical type in the central nervous system. Hypoxic-ischemic brain injury is relatively common in clinical neonates, mainly caused by intrauterine distress, asphyxia, and hypoxia in the perinatal period. A series of abnormal brain symptoms of the central nervous system may occur, and it is one of the leading causes of neonatal death[(1)]. Relevant data show that 80% of neonatal hypoxic-ischemic brain injury is caused by neonatal asphyxia, accounting for the third cause of neonatal death globally, which burdens families and society[(2)]. Therefore, it is essential to study the pathogenesis of hypoxic-ischemic brain injury in more depth and find safe and effective treatment methods to reduce neonatal mortality and disability rate.

At present, the pathogenesis of hypoxic-ischemic brain injury has not been fully clarified. However, it is generally believed to be related to the metabolic failure of neurons, free radical damage, dysfunction of cerebrovascular autonomic regulation, and cell

apoptosis[3]. Histone deacetylase (HDAC) is a class of enzymes that can change chromosome structure and regulate gene expression. Studies have confirmed that HDAC plays a crucial role in regulating nerve function[4]. Oxidative stress injury is the basis of many kinds of nervous system diseases, which can promote the degeneration of neurons and protect them by inhibiting oxidative stress injury. As one of the main components of Herba Leonurus, stachydrine improves hemorheology and antithrombosis and improves coronary blood flow, myocardial ischemia, and reduces blood viscosity in terms of diabetes and cardiovascular diseases[5]. Furthermore, studies have confirmed that stachydrine is an HDAC inhibitor[6]. Therefore, the purpose of this study was to analyse the protective effect of stachydrine on hypoxic-ischemic brain injury in neonatal rats by regulating HDAC activity, oxidative stress response, and neuronal inflammation.

Materials and methods

Reagents and instruments

The following reagents and instruments were implemented:

- Sue alkali water (Shanghai Xinyu Biological Technology Co., Ltd.)
- TUNEL Apoptosis Assay Kit (Emmett Technology Co., Ltd.)
- ELISA Kit (Shanghai Fusheng Industrial Co., Ltd.)
- BCA Protein Quantitative Kit (Shenyang Wanjie Biotechnology Co., Ltd.)
- DAB Color Development Kit (Shanghai Jizhi Biochemical Technology Co., Ltd.)
- Antigen Repair Solution (Qingdao Jieshikang Biotechnology Co., Ltd.)
- HDAC activity assay kit (HDAC activity assay kit)
- Acetylated protein H3, H4 antibody (Shanghai Yaji Biotechnology Co., Ltd.)
- Automatic biochemical analyser (Nanjing Baden Medical Co., Ltd.)
- Automatic autoclave (Hangzhou Nuoding Scientific Equipment Co., Ltd.)
- Optical Microscope (Shanghai Yiji Industrial Co., Ltd.)
- Magnetic stirrer (Shanghai Fuze Trading Co., Ltd.)
- Water Bath Shaker (Thermo Fisher Technology)
- Enzyme Label (Beijing Anmag Trading Co., Ltd.)
- Electrophoresis tank (Beijing Yiaobo Technology and Trade Co., Ltd.)
- Image Analysis System (Beijing Jiayuan Xingye Technology Co., Ltd.)
- UV spectrophotometer (Shanghai Zhenyu Biological Technology Co., Ltd.).

Treatment of experimental animals

Thirty-two 7-day-old newborn SD rats with a bodyweight of 12~16g were selected and purchased from Beijing Vitong Lihua Experimental Animal Technology Co., Ltd. The rats were fed day and night for 12 hours each in the animal experiment center of our hospital at 20~23°C and 60~70% humidity. The neonatal rats were divided into the control group, model group, stachydrine 5mg/kg and 10mg/kg groups, with eight rats in each group. Regarding the operation, chloral hydrate 0.3ml/100g anaesthetised rats were fixed on the aseptic operating table. Then, a 0.5 cm cut was made to the head starting from the upper left of the intersection of the midline of the neck and the root of both forelimbs to expose the carotid artery triangle. Next, the common carotid artery was separated and ligated. After cutting off the left common carotid artery, the skin was sutured and placed in the mother's nest for 2-3 h after recovery, and then placed in a sealed anoxic chamber for anoxic treatment for 2.5 h. Rats in the control group only received skin sutures after exposing the left common carotid artery without hypoxia treatment. After successful modeling, the model group was intraperitoneally injected with 5 ml/kg normal saline. The stachydrine groups were intraperitoneally injected with 5 mg/kg and 10 mg/kg stachydrine, respectively. After completing the treatment plan, the rats were deeply anaesthetised and euthanised for brain tissue.

Observation indexes

Evaluation of rat infarct area: The brain tissue sections of rats were placed in a PBS solution containing TTC, and the tissue sections were observed to be closed when stained in red. The infarct area of rats was calculated by computer image analysis software.

Apoptosis of brain tissue cells: The brain tissue was soaked in 4% paraformaldehyde and then treated with dehydration, paraffin impregnation, and TUNEL staining was performed. The color of apoptotic positive cells was brown-yellow. Five positive fields at high magnification (×400) were randomly selected from the sections, and the number

of positive cells in 300 cardiomyocytes in each field was counted. The average value was the apoptotic index. Two pathologists performed a double-blind analysis.

ELISA was used to detect HDAC activity, tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β), intercellular adhesion molecule (ICAM-1), and vascular endothelial cell adhesion molecule-1(VCAM-1). Serum levels of superoxide dismutase (SOD) and malondialdehyde (MDA) were determined by the barbital sulfate method. Glutathione peroxidase (GSH-Px) and total antioxidant capacity (T-AOC) were detected by chemical colorimetry.

The expression of acetylated proteins H3 and H4 were detected by Western blot. Brain tissues were added with RIPA lysate to lyse cells, and quantitative protein analysis was conducted. Protein samples were taken by SDS-polyacrylamide gel electrophoresis and transferred to the PVDF membrane. The protein samples were sealed with 5% skim milk powder, incubated with primary antibody, then incubated overnight in a refrigerator at 4°C, and incubated at room temperature with corresponding secondary antibody.

Statistical methods

Data in this study were analysed using the SPSS21.0 software package. Measurement data conforming to normal distribution were expressed by . Repeated measurement analysis of variance was used to compare multiple groups, SNK-Q test was used for comparison between two groups, and P<0.05 was considered statistically significant.

Results

Effect of stachydrine on infarct size in rats with neonatal hypoxic-ischemic brain injury

The cerebral infarction area in the cortical region of neonatal rats in the model group was significantly higher than that in the control group, and the difference was statistically significant (P<0.05). As seen in Figure 1, the cerebral infarct area in the cortical region of rats in 5 mg/kg and 10 mg/kg stachydrine groups was significantly lower than that in the model group, and the difference was statistically significant (P<0.05).

Effect of stachydrine on cell apoptosis in rats with neonatal hypoxic-ischemic brain injury

The apoptosis rate of brain tissue cells in the model group was significantly higher than that in the control group (P<0.05). Furthermore, the apoptosis rate of brain tissue cells in the 5 mg/kg and 10 mg/kg stachydrine groups was significantly lower than in the model group, and the difference was statistically significant (P<0.05), as shown in Figure 2.

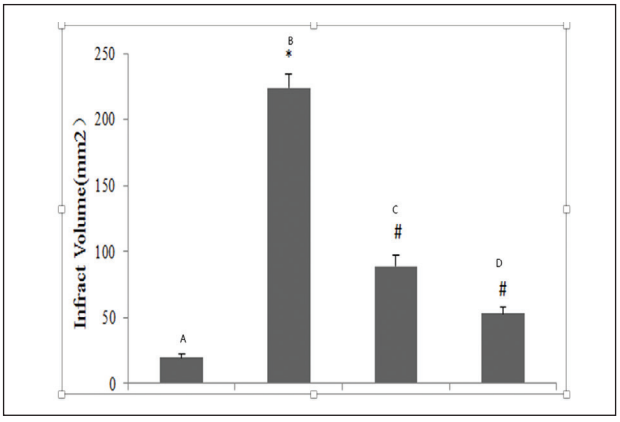

Figure 1: Effect of stachydrine on infarct size in rats with neonatal hypoxic-ischemic brain injury.
*Note: Compared with the control group, *P<0.05; Compare with the model group,#P<0.05.A, Control; B, Model; C, 5 mg/kg stachydrine; D,10 mg/kg stachydrine*

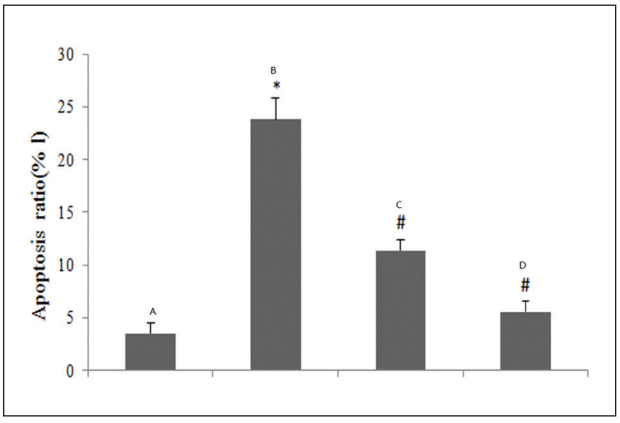

Figure 2: Effect of stachydrine on apoptosis of neonatal hypoxic-ischemic brain injury in rats.
*Note: Compared with the control group, *P<0.05; Compare with the model group,#P<0.05.*
A, Control; B, Model; C, 5 mg/kg stachydrine; D,10 mg/kg stachydrine

Effect of stachydrine on HDAC activity in brain tissue of rats with neonatal hypoxic-ischemic brain injury

The activity of brain HDAC in the model group was significantly higher than that in the control group (P<0.05). Additionally, in the water Sue alkali 5 mg/kg and 10 mg/kg dose group of brain tissue, the HDAC activity was significantly lower than the model group, with a statistically significant difference (P<0.05), as shown in Figure 3. Further

analysis showed that the expressions of acetylated proteins, H3 and H4, in brain tissue of rats in the 5 mg/kg and 10 mg/kg stachydrine groups were significantly higher than those in the model group (P<0.05).

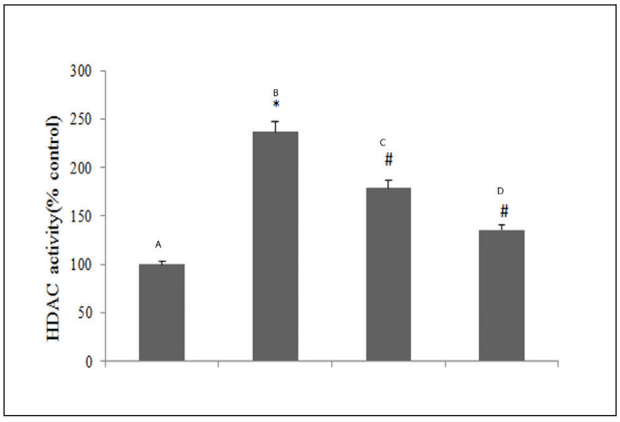

Figure 3: Effect of stachydrine on HDAC activity in brain tissue of rats with neonatal hypoxic-ischemic brain injury. A, Control; B, Model; C, 5 mg/kg stachydrine; D,10 mg/kg stachydrine.

Effects of stachydrine on oxidative stress indexes in rats with neonatal hypoxic-ischemic brain injury

The levels of SOD, GSH-Px, and T-AOC in brain tissue of rats in the model group were significantly lower than those in the control group. In comparison, the levels of MDA in the model group were significantly higher than those in the control group (P<0.05). The water Sue alkali 5 mg/kg, 10 mg/kg dose group of brain tissue SOD, GSH-px, and T-AOC level was higher than that of the model group, and the MDA level significantly lower than the model group (P<0.05). See table 1.

Group	SOD (kU/g)	MDA (mol/mg)	GSH-Px (kU/g)	T-AOC (kU/g)
Control group	32.45±7.10	1.02±0.22	44.80±8.39	1.57±0.15
Model group	11.89±2.84*	3.16±0.35*	10.45±3.42*	0.33±0.14*
5mg/kg stachydrine group	25.56±4.55#	2.05±0.52#	32.26±7.19#	0.83±0.19#
10mg/kg stachydrinestachylostachine group	29.51±3.02#	1.46±0.40#	36.15±8.79#	1.15±0.28#
F	29.95	45.70	32.39	55.96
P	<0.001	<0.001	<0.001	<0.001

Table 1: Effect of stachydrine on oxidative stress indexes of neonatal hypoxic-ischemic brain injury rats.
*Note: Compared with the control group, *P<0.05; Compare with the model group,#P<0.05.*

Effect of stachydrine on inflammatory factors in neonatal hypoxic-ischemic brain injury in rats

The levels of TNF-α, IL-1β, ICAM-1, and VCAM-1 in the brain tissue of the model group were significantly higher than those in the control group (P<0.05). Further, the levels of TNF-α, IL-1β, ICAM-1, and VCAM-1 in 5 mg/kg and 10 mg/kg stachydrine groups were significantly lower than those in the model group (P<0.05). See table 2.

Group	TNF-α (pg/mg)	IL-1β (pg/mg)	ICAM-1 (pg/mg)	VCAM-1 (pg/mg)
Control group	8.63±7.87	64.72±15.24	141.43±73.40	23.02±7.72
Model group	17.76±5.53*	119.05±46.12*	235.18±65.51*	43.60±11.68*
5mg/kg stachydrine	10.67±6.30#	82.62±46.05#	162.13±43.16#	32.71±13.79#
10mg/kg stachydrine	8.88±3.42#	75.88±26.62#	145.12±41.66#	24.71±13.82#
F	4.08	3.41	4.61	4.90
P	0.016	0.031	0.010	0.007

Table 2: Effect of stachydrine on inflammatory cytokines in rats with neonatal hypoxic-ischemic brain injury.
*Note: Compared with the control group, *P<0.05; Compare with the model group,#P<0.05.*

Discussion

Hypoxic-ischemic brain injury is one of the primary causes of neonatal death and neurological complications, including mental retardation, growth and development delay, visual and hearing impairment, and epilepsy. This disease's early and middle pathophysiological changes manifest by free radical generation and excitatory toxicity. After that, events like white matter demyelination injury, excessive release of neurotransmitters, increased blood-brain barrier permeability, neuroinflammatory response, and cell apoptosis occur. This is followed by neuroangiogenesis, glial cell proliferation, microglial cell activation, and other manifestations in the later stage[7-8].

Studies have found that oxidative stress plays an essential role in the pathological process of hypoxic-ischemic brain injury. The balance between oxidative and antioxidant factors is broken, resulting in the excessive release of reactive oxygen free radicals, which can further lead to neuronal degeneration and death[9]. Hypoxic-ischemic brain injury can reduce histone acetylation, especially histone H3 acetylation, which in turn increases the inflammatory response of microglia. Studies have been conducted on HDAC to control different protein gene expressions and the function of enzymes that can have a catalytic effect on histone acetyl and cause corresponding gene expression.

These studies have confirmed that the HDAC inhibitor can correct the central nervous pathological state under the condition of chromosomes, inhibition, reducing inflammatory reaction, neuron apoptosis, and cell excitability and toxicity, which have the effect of nerve protection[10].

In recent years, natural products with a neuroprotective effect to improve cerebral ischemic

injury have become a hot spot of clinical research. Furthermore, stachydrine is the most important in the pharmacological effects of Leonurus, which has a variety of pharmacological activities on the uterus, heart, blood vessels, kidneys, and other organs[11]. For example, stachydrine can reduce postpartum bleeding and promote uterine repair. For the blood system, stachydrine protects vascular endothelium, dilating vessels, anticoagulation, and anti-platelet aggregation. For the kidneys, stachydrine has a diuretic effect and protects the kidneys. Moreover, in the cardiovascular system, stachydrine can resist myocardial ischemia, reduce the amount of myocardial cell necrosis, and protect myocardial cells[12].

In this study, the protective effect of stachydrine on the nervous system of the experimental animal model was studied by establishing the neonatal rat model of hypoxic-ischemic brain injury. The results showed that the cerebral infarct area and apoptosis rate in the cortical region of rats in the stachydrine 5 mg/kg and 10 mg/kg groups were significantly lower than those in the model group ($P<0.05$). Therefore, it can be concluded that stachydrine has a neuroprotective effect on hypoxic-ischemic brain injury rats, and there is a dose-dependent effect. The reason may be related to the effect of stachydrine on vasodilation, increasing blood flow, and decreasing blood viscosity. In addition, scholars have found that stachydrine can down-regulate the expression of ER stress-related apoptotic proteins and thus inhibit cell apoptosis[13]. Recent studies have shown that HDAC inhibitors in hypoxic-ischemic brain injury can regulate histone modification, maintain the relationship between histone acetylation and deacetylation, and act on multiple links in the process of brain injury, thereby alleviating brain tissue injury[14].

This study also found that stachydrine can decrease HDC activity and increase H3 and H4 protein expression, providing evidence to support its neuroprotective effect. Furthermore, this study found that SOD, GSH-Px, and T-AOC levels in brain tissue of water Sue alkali 5 mg/kg and 10 mg/kg dose group were higher than that of the model group. Also, the MDA level was significantly lower than the model group ($P<0.05$), indicating that stachydrine can reduce the oxidative stress response of rats with brain injury and play a protective role in nerve cells. Some foreign scholars established the endothelial cell injury model and gave stachydrine intervention. The results showed that the activity of endothelial cells and the reduction of SOD and GSH-Px activities could be significantly reversed, and the expression level of MDA could be reduced (15). The results showed that the levels of TNF-α, IL-1β, ICAM-1, and VCAM-1 in 5 mg/kg and 10 mg/kg stachydrine groups were significantly lower than those in the model group ($P<0.05$). It was concluded that stachydrine can reduce the inflammatory response in rats with hypoxic-ischemic brain injury, which may be one of the mechanisms of stachydrine in neuroprotection.

In conclusion, stachydrine can improve the injury degree of neonatal rats with hypoxic-ischemic brain injury and has an apparent protective effect, which may be realised by inhibiting HDAC activity and regulating oxidative stress response and neuronal inflammatory response.

References

1) Galinsky R, Davidson JO, Dean JM, Green CR, Bennet L, et al. Glia and hemichannels: key mediators of perinatal encephalopathy. Neural Regen Res. 2018 Feb; 13(2): 181-189.
2) Diggikar S. Therapeutic Hypothermia using Phase Changing Material in Indian Neonates. Indian Pediatr. 2018 Aug 15; 55(8): 710-711. PMID: 30218529.
3) Lemmon ME, Wagner MW, Bosemani T, Carson KA, Northington FJ, et al. Diffusion Tensor Imaging Detects Occult Cerebellar Injury in Severe Neonatal Hypoxic-Ischemic Encephalopathy. Dev Neurosci. 2017; 39(1-4): 207-214.
4) Ali MN, Choijookhuu N, Takagi H, Srisowanna N, Nguyen Nhat Huynh M, et al. The HDAC Inhibitor, SAHA, Prevents Colonic Inflammation by Suppressing Pro-inflammatory Cytokines and Chemokines in DSS-induced Colitis. Acta Histochem Cytochem. 2018 Feb 27;51(1):33-40.
5) Zhao L, Wu D, Sang M, Xu Y, Liu Z, et al. Stachydrine ameliorates isoproterenol-induced cardiac hypertrophy and fibrosis by suppressing inflammation and oxidative stress through inhibiting NF-ϰB and JAK/STAT signaling pathways in rats. Int Immunopharmacol. 2017 Jul; 48: 102-109.
6) Ganzer PD, Loeian MS, Roof SR, Teng B, Lin L, et al. Dynamic detection and reversal of myocardial ischemia using an artificially intelligent bioelectronic medicine. Sci Adv. 2022 Jan 7; 8(1):eabj5473.
7) Ramirez A, Peyvandi S, Cox S, Gano D, Xu D, et al. Neonatal brain injury influences structural connectivity and childhood functional outcomes. PLoS One. 2022 Jan 5; 17(1): e0262310.

8) Lee BS, Jung E, Lee Y, Chung SH. Hypothermia decreased the expression of heat shock proteins in neonatal rat model of hypoxic ischemic encephalopathy. Cell Stress Chaperones. 2017 May; 22(3): 409-415.
9) Soliman MM, Gaber A, Alsanie WF, Mohamed WA, Metwally MMM, et al. Gibberellic acid-induced hepatorenal dysfunction and oxidative stress: Mitigation by quercetin through modulation of antioxidant, anti-inflammatory, and antiapoptotic activities. J Food Biochem. 2022 Jan 5: e14069.
10) Booth L, Roberts JL, Poklepovic A, Avogadri-Connors F, Cutler RE, et al. HDAC inhibitors enhance neratinib activity and when combined enhance the actions of an anti-PD-1 immunomodulatory antibody in vivo. Oncotarget. 2017 Oct 9;8(52):90262-90277.
11) Eyre DW, Taylor D, Purver M, Chapman D, Fowler T, et al. Effect of Covid-19 Vaccination on Transmission of Alpha and Delta Variants. N Engl J Med. 2022 Jan 5.
12) Xiong Y, Cao XY, Liu BY, Dai YQ, Zhou HJ, et al. Exploring the mechanism of Buxue Yimu Pill on hemorrhagic anemia through molecular docking, network pharmacology and experimental validation. Chin J Nat Med. 2021 Dec; 19(12): 900-911.
13) Chen M, Yang D, Hu X, Jiang G, Li T, et al. Stachydrine hydrochloride inhibits osteoclastogenesis by regulating the NF-κB and p38 signaling pathways to alleviate postmenopausal osteoporosis. Biochem Biophys Res Commun. 2021 Jan 19; 542:1-8.
14) Servillo L, D'Onofrio N, Giovane A, Casale R, Cautela D, et al. The betaine profile of cereal flours unveils new and uncommon betaines. Food Chem. 2018 Jan 15; 239: 234-241.
15) Swan HB, Deschaseaux ESM, Jones GB, Eyre BD. The relative abundance of dimethylsulfoniopropionate (DMSP) among other zwitterions in branching coral at Heron Island, southern Great Barrier Reef. Anal Bioanal Chem. 2017 Jul; 409(18): 4409-4423.

Corresponding Authors:
Haiyang Lin
No. 9 Jinsui Road, Tianhe District, Guangzhou City, Guangdong Province, China
Email: x4020w@163.com
(China)