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Research Article

Study of the Extraction and Hypolipidemic Mechanism of Steviosides from *Stevia rebaudiana* Leaves

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Abstract: In order to understand the hypolipidemic mechanism of steviosides from *Stevia rebaudiana* leaves, we optimised ratio of solid to liquid, extraction temperatures, extraction times, time to obtain optimal extraction conditions. Under the optimal condition (solid to liquid 1:20, extraction temperature 80°C, and extraction times 4h, time 4), the experimental yield of steviosides was gave the best results (8.637±0.27%). The SVS-2 were obtained by a DEAE-52 cellulose column and Sephadex G-15 column chromatography. The SVS-2 was characterised through FT-IR spectroscopy, NMR spectroscopy, HPLC-PAD-MS analysis by comparison with a stevioside standard. *In vitro* test, the steviosides can decrease liver TC, TG and serum TG, TC level and increased swimming time of rats fed with high-fat diets. Therefore, the results suggest that steviosides had a high hypolipidemic activity and could be used as a potential therapeutic agent for hyperlipidemia.

Keywords: Extraction, forced swimming test, hypolipidemic, mice, stevioside, *S. rebaudiana*

INTRODUCTION

Humans like sweet foods and a sedentary life style, this has led to an fast increase in obesity and diabetes. *Stevia rebaudiana* (*S. rebaudiana*) is a perennial shrub of the Asteraceae (Compositae) family initially grown in South America (Palazzo *et al*., 2011). The main sweet component in the leaves of *S. rebaudiana* is stevioside and dulcoside A, steviolbioside and rebaudiosides A. Stevioside have a sweetness of 300-400 times greater than solutions containing 0.4% sucrose (Kroyer, 2010). The use of stevioside as a natural sweeteners has been approved in USA since 2008 (Li *et al*., 2013), stevioside and extracts prepared from the leaves of *S. rebaudiana* have been widely used around the world as sweetening agents, taste modifiers and sugar substitutes; in addition, there have been no adverse effects reported from its use by humans (Brahmachari *et al*., 2011).

The extraction procedure by hot water has been used as a classical extraction method (Dacome *et al*., 2005). Therefore, in this study, steviosides were isolated and purified from *S. rebaudiana* leaves by water and column chromatography separation. The FT-IR, NMR and HPLC-PAD-MS analysis revealed their preliminary characteristics.

Hyper lipidemia is a major risk factor for atherosclerosis and cardiovascular diseases (Park, 2009; Schwingshackl and Hoffmann, 2014). Moreover, stevioside have several physiological activities such as lowering blood pressure (Chan *et al*., 2000), lowering blood sugar (Gregersen *et al*., 2004) and anti-tumor (Yasukawa *et al*., 2002). Stevioside have a direct effect on glucagon secretion as well (Hong *et al*., 2006). Our work was aimed to extract steviosides using the optimized process and investigate the polysaccharide characteristic. Further more to determine the effect of steviosides on the TC, TG in liver and serum of mice.

MATERIALS AND METHODS

Materials and equipment: *S. rebaudiana* leaves were provided by Yancheng Xiaguang Stevioside Trading Company, China and stored at 4°C in refrigerator. Samples of steviosides, Tianjin Meilun Medical Co., Ltd. TG and TC were purchased from Nanjing Jiancheng Bioengineering Institute (Jiangsu, China). All other chemicals were reagent grade.

Test animals: Female mice, with weights 25±3g, were purchased from the Institute of Zoology, Chinese Academy of Medical Sciences (Beijing, China) used in

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Table 1: Factors and levels for orthogonal test

	Levels			
Variable	1	2	3	4
(A) Atio of solid to liquid	1 : 5	1 : 10	1 : 15	1: 20
(B) Extraction temperature (°C)	20	40	60	80
(C) Extraction times	1	2	3	4
(D) Time (h)	1	2	3	4

this study. The mice were individually housed in metabolic and kept in an air-conditioned room at 22±1°C on a 12h light-dark cycle. All animal protocols were performed in compliance with the Chinese legislation on the use and care of laboratory animals and were approved by the university committees for animal experiments.

Mice were fed with basic diet for 1 week in the experimental environment before the experiments to adapt to the environment. The mice were randomly divided into three groups (10 mice per group): Normal Group (NG), received basic diet; High-Fat Group (HFG), received high-fat diet containing 10% (w/w) lard, 15% (w/w)egg yolk powder, 1%(w/w) cholesterol and commercial diet to 100% (w/w); Steviosides Group (SG), received high-fat diet plus gavaged 0.2 mL 5% (w/w) steviosides.

Extraction procedure: In order to evaluate the effect of extraction conditions on the yields of steviosides, experiments were done at ratio of solid to liquid (1/5,1/10,1/15,1/20), different extraction temperatures(kept at 40, 50, 60, 70, and 80°C with a water circulation system), extraction times (1,2,3,4) and time(1h, 2h, 3h,4h). An orthogonal L_{16} (4^5) design was used to investigate the optimal extraction conditions of steviosides. The independent variables in this experiment were ratio of solid to liquid (X1), extraction temperature (X2), extraction times (X3) and time (X4). Table 1 shows the detailed experimental conditions for the extraction of steviosides from *S. rebaudiana* leaves. The supernatant was added 95% ethanol again to a final concentration of 70% and kept at 4°C overnight, precipitated polysaccharides was collected by centrifugation.

Isolation and purification of steviosides: Filtrate was applied to a DEAE-52 cellulose column (4.0×100cm), stepwise eluted with equilibrated with 0, 0.1, 0.2, 0.3 and 0.4mol/L NaCl at a flow rate of 2mL/min. The eluent(4mL/tube) was collected and each major fraction obtained, concentrated, furtherly applied on a Sephadex G-15 with deionized water at a flow rate of 1mL/min. The fractions obtained were combined according to the total carbohydrate content measured by the phenol-sulfuric acid method. The major fraction obtained was concentrated, dialyzed and lyophilized.

FT-IR spectroscopy analysis: The FT-IR spectrum of the extractive was determined using a NicoletiS50 FT-IR spectrometer (Thermo Nicolet Co., USA) in the wave number range of 400-4000cm^{-1} with KBr pellets.

NMR spectroscopic analysis: The extractive was dissolved in 0.5mL of D_2O (99.9%), and NMR spectra were recorded on a Bruker Acsend 600 spectrometer operating at 400.15 MHz (1H) and 100.57 MHz (13C). The water signal at 5ppm in 1H NMR was suppressed.

H`PLC-PAD-MS analysis: The HPLC-PAD-MS analysis was used a LXQ linear ion trap mass spectrometer equipped with an electrospray ion source(ESI) and a photodiode array detector(PAD), controlled by XCalibur software(Thermo Fisher Scientific, Basel, Switzerland). The ESI-MS settings were as follows: spray voltage: 3kV, capillary temperature: 325°C, capillary voltage: 37V, sheath gas: 40 Arbitrary Units (AU), auxiliary gas: 10AU. All the data were acquired in positive mode, and the scan range was set from m/z 100 to 1000am. A Silgreen ODS C18 (4.6mm×750mm) was used and kept at 30°C, and the mobile phase consisted of 20mM ammonium acetate-acetonitrile (78:22, v/v) with a flow rate of 0.25mL/min.

Liver and serum lipids of mice: At the sixth week, mice were given diethylether after fasting for 18 h. Blood was collected, and plasma was obtained by centrifugation at 3000×g for 10min. Plasma samples were stored at −20°C for further analysis.

TC, TG in liver and serum were measured with commercial assay kits.

Forced Swimming Test (FST): The FST was conducted as described by Can *et al*. (2013). In this test, mice were individually forced to swim in an open cylindrical container (diameter 10cm, height 25cm), containing 19cm of water at 25±1°C.

At the sixth week, mice were placed in the apparatus swiming for 6min and the behaviors were monitored. Each mice was judged to be immobile when it ceased struggling and remained floating motionless in the water, making only those movements necessary to keep its head above water.

Statistical analysis: All the data were exhibited as three replicate determinations. Difference was considered to be significant when $p<0.05$. Statistical analysis involved use of the Origin Pro software package8.5 and SPSS13.0.

RESULTS AND DISCUSSION

Optimization of the extraction parameters of steviosides: Ratio of solid to liquid (X1), extraction

Table 2: Analysis of $L_{16}(4)^5$ test results

No.	(A) Atio of solid to liquid	(B) Extraction temperature (°C)	(C) Extraction times	(D) Time (h)	(E) Blank	Extraction yield (%)
1	1	1	1	1	1	5.102
2	1	2	2	2	2	5.163
3	1	3	3	3	3	7.034
4	1	4	4	4	4	8.264
5	2	1	2	3	4	6.571
6	2	2	1	4	3	6.946
7	2	3	4	1	2	8.568
8	2	4	3	2	1	8.194
9	3	1	3	4	2	7.747
10	3	2	1	3	1	8.291
11	3	3	4	2	4	6.313
12	3	4	2	1	3	6.915
13	4	1	4	2	3	7.536
14	4	2	3	1	4	7.692
15	4	3	2	4	1	6.915
16	4	4	1	3	2	6.577
K1	25.55	26.94	26.90	28.26	28.49	
K2	30.26	28.08	25.55	27.19	28.03	
K3	29.25	28.81	30.65	28.46	28.41	
K4	28.70	29.93	30.66	29.85	28.83	
k1	6.388	6.735	6.725	7.065	7.123	
k2	7.565	7.020	6.388	6.798	7.008	
k3	7.313	7.203	7.663	7.115	7.103	
k4	7.175	7.483	7.665	7.463	7.208	
R	1.178	0.748	1.278	0.665	0.200	

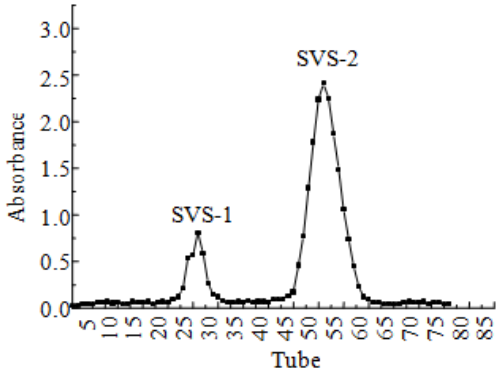

Fig. 1: Fractionation of filtrate by a DEAE-52 cellulose column

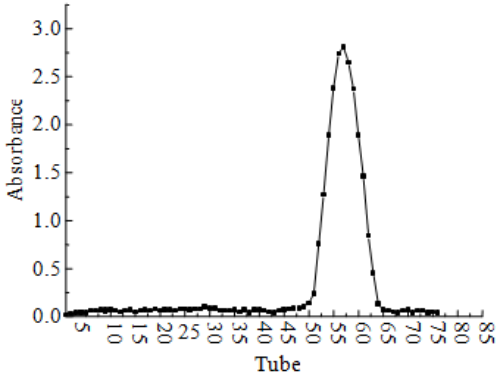

Fig. 2: Fractionation of SVS-2 by Sephadex G-15 column chromatography

temperature (X2), extraction times (X3), time (X4) are considered to be the most important factors that affect the yield (%) of the steviosides (SVS). Independent variables with four variation levels are listed in Table 1. All selected factors were examined using an orthogonal $L_{16}(4^5)$ test design. The analysis results of orthogonal test, performed by statistical software SPSS13.0, are presented in Table 2. The maximum extraction yield of the SVS was 8.568%. However, we cannot select the best extraction conditions only based on these outcomes, the K, k and R values were calculated and listed in Tab.2. The factors influence the yield (%) of the SVS were listed in an increasing order as follows: C>A>B>D according to the R value. So the maximum yield of the SVS was obtained when extraction time, extraction temperature, number of extraction and water to raw material ratio were C2A4B4D4(2; 1:20; 80°C; 4h), respectively. Through confirmatory test, we get the high yield and quality stevioside, with a yield (%) of 8.637±0.27%.

Purification of SVS and physicochemical property: The SVS were separated through a DEAE-52 cellulose column, affording two independent elution peaks and named SVS-1, SVS-2 (Fig. 1) as detected by the phenol-sulfuric acid colorimetric method. The main fraction SVS-2 was collected, concentrated and purified by Sephadex G-15 (Fig. 2). The SVS-2 were collected, concentrated, dialyzed and lyophilized for further analysis.

The FT-IR spectra of SVS-2 were shown in Fig. 3, broadband around 3353/cm exhibited O-H stretch

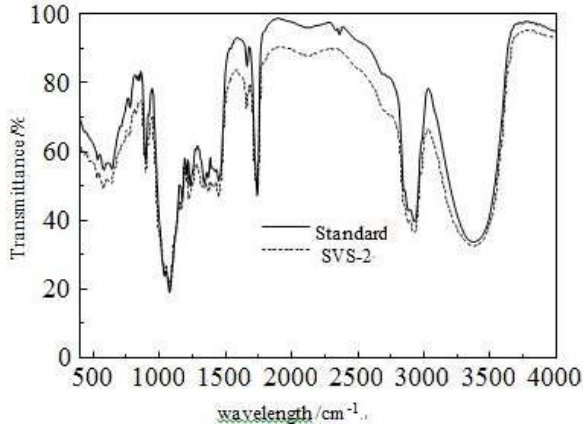

Fig. 3: FT-IR spectra of SVS-2

vibration, a weak peak at 291/cm was assigned to C-H stretch vibration, a peak at 1730/cm was assigned to C = O stretch vibration, a peak at 1032/cm was assigned to C-H-C stretch vibration, a peak at 886cm^{-1} was assigned to =CH2 stretch vibration.

HPLC-PAD-MS chromatogram (Fig. 4) indicated that the Mw of polysaccharide is 804.3.

The structural feature of SVS-2 was further analyzed by the NMR spectra. The spectra of 1H and 13C NMR were shown in Fig. 5 and 6. 1H-NMR(Pyridine-d5, 400MHz): 0.76(1H, brt, J = 12.9 Hz, H-1), 1.76(1H, d, J = 12.5 Hz, H-1), 2.26-2.21(1H, m, H-2), 1.46(1H, d, J = 12.7 Hz, H-2), 1.04-1.01(1H, m, H-3), 2.36(1H, d, J = 12.6 Hz, H-3), 1.05(1H, d, J = 12.2Hz, H- 5), 2.47-2.45(1H, m, H-6), 1.92(1H, d, J = 13.2 Hz, H-6), 1.32 (2H, m, H-7), 0.89(1H, d, J = 4.2Hz, H-9), 1.69(2H, d, J = 4.2 Hz, H-11), 2.27-2.21 and 2.01-1.91(1H, m, H-12), 2.65(1H, d, J = 11.6Hz, H-14), 1.82(1H, d, J = 11.7Hz, H-14), 2.05-2.01(2H, m, H-15), 5.64(1H, s, H-17), 5.01(1H, s, H-17), 1.31(3H, s, H-18), 1.25(3H, s, H-20). 13C-NMR(Pyridine-d5,100 MHz): 42.0(C-1), 20.7(C-2), 39.7(C-3), 45.3(C-4), 58.6(C-5), 23.4(C-6), 43.0(C-7), 43.8(C-8), 55.3(C-9), 41.1(C-10), 21.9(C-11), 38.2(C-12), 87.8(C-13), 45.8(C-14), 49.0(C-15), 155.4(C-16), 105.9(C-17), 29.6(C-18), 178.3(C-19), 16.8(C-20). All the results was consistent with the basic characteristic of steviosides.

Liver TC, TG and serum TG, TC level: A detailed investigation of mice liver TC, TG and serum TG, TC level were listed in Table 3. Though the liver TC, TG and serum TG, TC levels of the steviosides group were lower than those of the high-fat group, which suggested that steviosides can reduce the liver cholesterol level in mice fed high-fat diet. Lin and Lin-Shiau (2006) found that tea polyphenols can reduce the absorption of cholesterol and triglycerides. The high level of serum triglycerides is generally considered as a risk factor for cardiovascular diseases (Ntchapda *et al*., 2015). Table 4 show the behaviors of mice subjected to the FST. The result showed that steviosides can increase the swimming time in the mice FST when compared to the model group. This indicate that steviosides may play a role through inhibiting the hepatic biosynthesis of cholesterol.

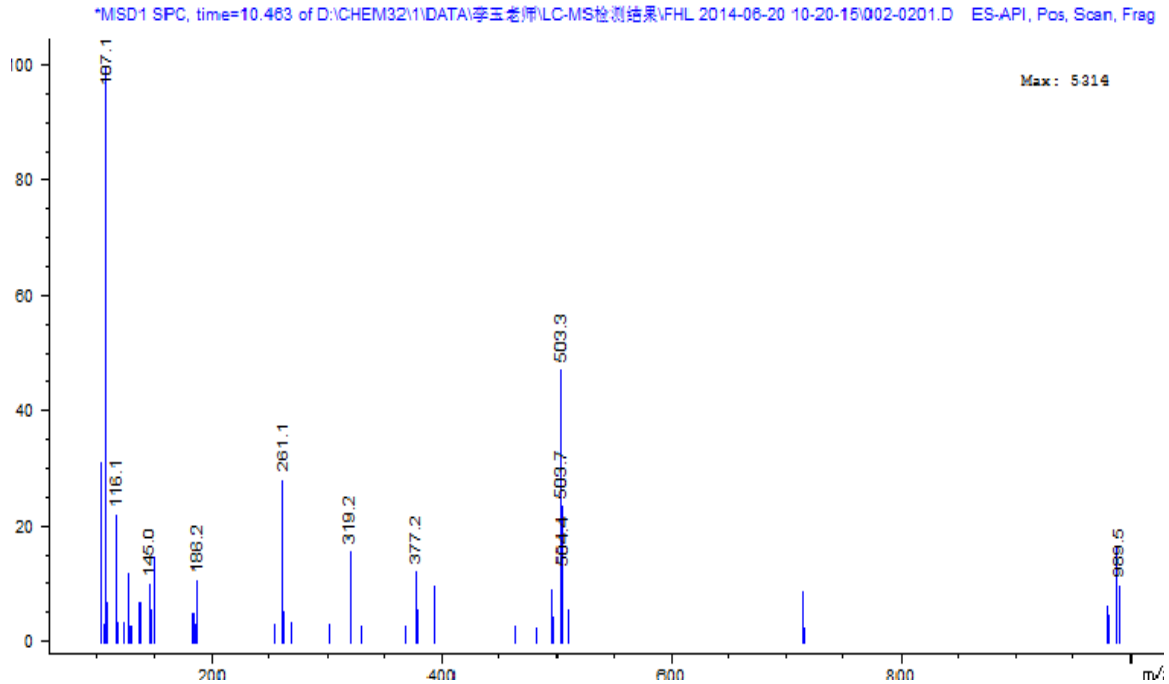

Fig. 4: HPLC-PAD-MS of SVS-2

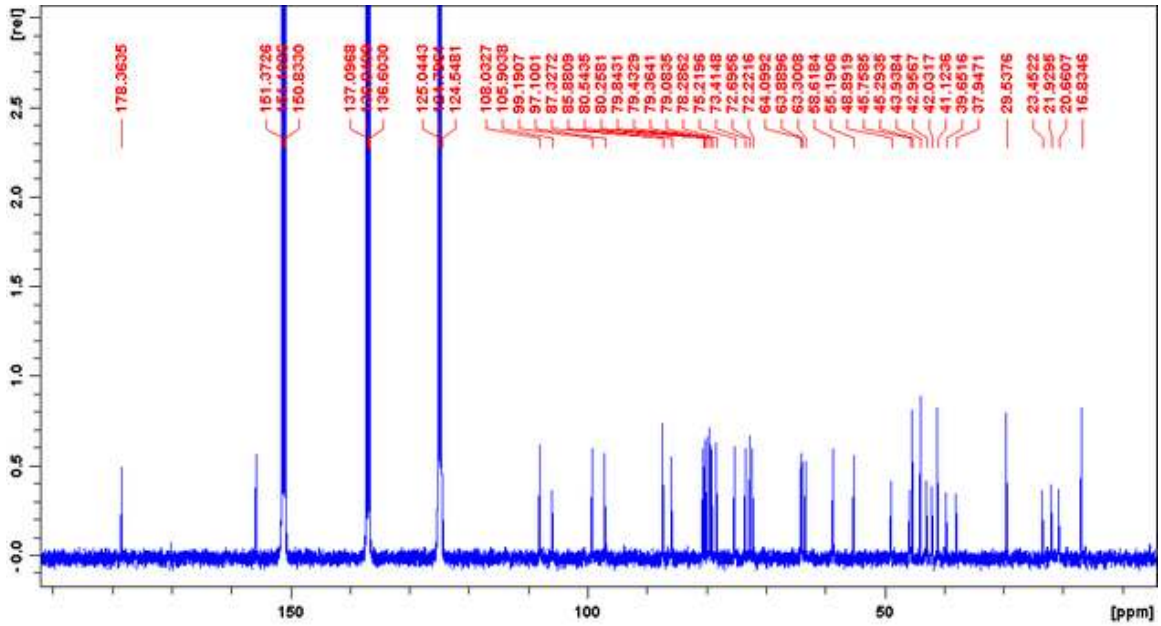

Fig. 5: The spectra of 13C NMR

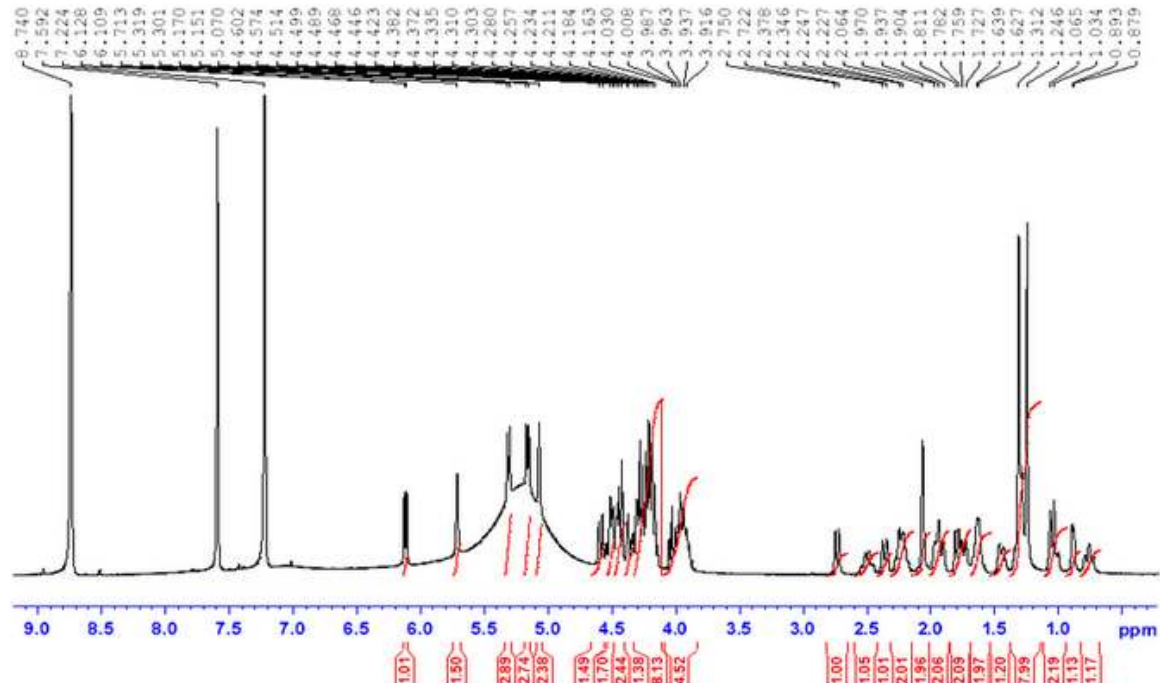

Fig. 6: The spectra of 1H NMR

Table 3: The liver TC, TG and serum TG, TC of rats fed on high fat, high fat plus steviosides, and normal fat diets after six weeks of diets

Groups	Serum TC	Serum TG	Liver TC	Liver TG
NG	2.72±0.17	1.15±0.11	7.81±0.159	1.82±0.15
HFG	4.14±0.11**	1.69±0.16**	10.52±0.62**	2.73±0.13**
SG	3.07±0.09$^{\#\#}$	1.36±0.08$^{\#}$	10.33±0.11	2.16±0.13$^{\#\#}$

Values are means±S.E.M., n = 5, *, #p<0.05, **, ##p<0.01.*: compared with the control group; #: compared with the model group

Table 4: Effects of steviosides on weight-loading swimming time

Group	NG	HFG	SG
Swimming time	156.63±9.62	88.97±6.75**	134.67±11.66$^{\#\#}$

Values are means±S.E.M., n = 5, *, #p<0.05, **, ## p<0.01.*: compared with the control group; #: compared with the model group

CONCLUSION

In this study, an optimization process was employed to extract steviosides from *S.rebaudiana* leaves. The optimal extraction conditions for the steviosides were as follows: solid to liquid 1:20, extraction temperature 80°C, and extraction time 4h, time 4, respectively. Under the optimal condition, the experimental yield of steviosides was 8.637±0.27%,which was close to the predicted value(8.6%).The purified steviosides was obtained by a DEAE-52 cellulose column and Sephadex G-15 column chromatography. Furthermore, *in vitro* test, the steviosides can decrease liver TC, TG and serum TG, TC level and increase swimming time of mice fed with high-fat diets. The results presented in this study provide a reference for the exploration of potential hypolipidemic from functional foods and further studies are essential to evaluate hypolipidemic activity *in vivo* and elucidate the potential hypolipidemic mechanism.

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