Reviewer #1：

1. Current report demonstrated the potential mechanisms for hypoglycemic effects of Lomatogonium rotatum (LR) in type-2 diabetic rats (T2D). I like to give the following comments.

Response 1: Thanks very much for the reviewer's comments! We appreciate the time and effort that the reviewer dedicated to providing feedback on our manuscript and are grateful for the insightful comments on and valuable improvements to our paper. We are incorporated most suggestions made by the reviewer. Those changes are highlighted within the manuscript as marked in red. Please see below, in red, for a point-by-point response to the reviewer's comments and concerns.

2. Collection and extraction of LR must describe in detail.

Response 2: Thanks for the reviewer's comment! As suggested, we added LR collection and extraction methods as follows:

Line 67-72: LR was collected from Xilinhaote grassland, Inner Mongolia, China. Five kilograms of LR was washed, dried and then crashed into powder. LR powder was extracted three times with 95% ethanol for 3 hours each time. The extract was combined and concentrated under vacuum at 60℃ and then freeze-dried. Carboxymethylcellulose sodium salt (CMC-Na) solution was used to dissolve the LR extract and the LR suspension was gavaged to animals at 0.5g/kg, 2.5g/kg, and 5g/kg according to the previous report [16].

[16] Bao S.; Wang X.; Ma Q.; Wei C.;, Nan J.; Ao W. Mongolian medicine in treating type 2 diabetes mellitus combined with nonalcoholic fatty liver disease via FXR/LXR-mediated P2X7R/NLRP3/NF-κB pathway activation. Chin Herb Med. 2022,14(3):367-375. doi: 10.1016/j.chmed.2022.06.003.

3. Induction of animal model and the treated dose of 0.5g/kg, 2.5g/kg, and 5g/kg from LR extract must follow the previous report(s).

Response 3: Thanks for the reviewer's advice! The reference dosage of STZ in animal model induction and the reference of dose ranges of LR were added in the main text as the reviewer's advice.

[16] Bao S, Wang X, Ma Q, Wei C, Nan J, Ao W. Mongolian medicine in treating type 2 diabetes mellitus combined with nonalcoholic fatty liver disease via FXR/LXR-mediated P2X7R/NLRP3/NF-κB pathway activation. Chin Herb Med. 2022 Jul 19;14(3):367-375. doi: 10.1016/j.chmed.2022.06.003. PMID: 36118003; PMCID: PMC9476729.

[17] Furman BL. Streptozotocin-Induced Diabetic Models in Mice and Rats. Curr Protoc. 2021 Apr;1(4):e78. doi: 10.1002/cpz1.78. PMID: 33905609.

4. In Figure 2, change in plasma insulin by LR was not same as that of GLP-1. Why? GLP-1 is known to promote insulin secretion.

Response 4: Thanks very much for the reviewer's insightful comments! With regards to insulin and GLP-1 levels in serum samples, actually they have a similar trend of reduction in the model group and elevation in LR and metformin groups. However, as the reviewer pointed out, the level of insulin was significantly elevated at 2.5g/kg and 5g/kg, while GLP-1 only increased at 5g/kg but not at 2.5g/kg dose. We speculate the following two reasons: First, 2.5g/kg dose of LR treatment may not be effective enough to transit to the target organ and stimulate the endocrine cells to secrete GLP-1 (although there is a slight numerically increase at 2.5g/kg dose). Second, GLP-1 may have a minor degradation during the experimental operation. From our knowledge and experiences, GLP-1 is an unstable blood hormonal peptide that released by endocrine cells of the ileum and the colon in response to the presence of drug molecules or nutrients. If not appropriately handle or store

the samples, GLP-1 can be degraded in the blood samples shortly. Hope our reply and speculation can be acceptable, thank you!

5. Figure legends in Figure 4 must show in clear. Direction of pathologist is required to histological identification.

Response 5: Thanks for the reviewer's advice! Figure 4 graphs were replaced with high quality images and the legend was revised as the reviewer's suggestion. Histological identification were supervised by a pathologist Dr. Pan Yipeng from (Email: panicpan@hainmc.edu.cn) the Second Affiliated Hospital of Hainan Medical University. Thank you!

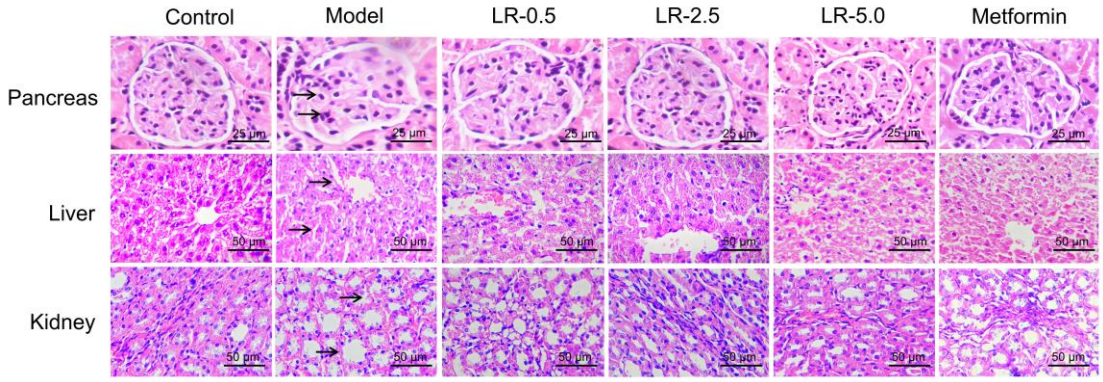


Figure 4. Effects of LR on histological changes of pancreas, liver, and kidney tissues in STZ-induced diabetic rats model. Arrows indicate: β-cell vacuolation and granulation in the pancreas; impaired central vein and steatosis in the liver; renal lesions and glomerular hypertrophy in the kidney.

6. In line 191, "Untargeted metabolomics analysis" shall be deleted.

Response 6: Thanks for the reviewer's suggestion! We have deleted it as advised. Thanks!

7. Machine learning of 236 metabolite annotations in serum samples must describe in detail.

Response 7: Thanks for the reviewer's advice! Raw data processing and

metabolite annotations approaches were described in detail in M&M section as below：

Line 138-147: After obtaining the raw data, the ChromaTOF software was used to automatically export the original GC-TOF/MS data to XploreMET (Metabo-Profile Biotechnology, Shanghai, China). This allowed for automated baseline denoising and smoothing, peak picking and deconvolution, creating a reference database from the pooled QC samples, metabolite signal alignment, missing value correction and imputation, metabolite identification, as well as data preprocessing (normalization and standardization). Then, for statistical analysis, all data were transformed into comparable data vectors. The standard deviation of the experimental measures was scaled and applied to each result, which was then mean-centered. The XploreMET software was used to carry out principal component analysis (PCA) and orthogonal partial least-square discriminant analysis (OPLS-DA). The sum of squares of the PLS weights was weighted using the value of the variable importance in the projection (VIP). The Kyoto Encyclopedia of Genes and Genomes looked at the metabolic process of many metabolites (KEGG).

8. Data in Table 1 are hard to follow. Please describe each in detail.

Response 8: Thanks for the reviewer's suggestion! Table 1 was rearranged and described as below.

Line 248-257: The representative differential metabolites obtained from the key altered pathways are illustrated in Table 1. The levels of Mevalonic acid-5P, D-Proline, L-Lysine, Taurine, Pyridoxal, Marshrin, Honyucitrin, Isoliquiritigenin, 1H-Indole-2,3-dione, Oxychlordane, Phosphorylcholine, Se-Adenosylselenohomocysteine, 1-Methyladenosine, LysoPE(0:0/18:3(6Z,9Z,12Z)), PE(20:4(8Z,11Z,14Z,17Z)/P-16:0), Bakers yeast extract, Ecgonine methyl ester showed a significant decrease in the model group in comparison with the control group. In contrast, LR treatment

dramatically increased the above metabolites in the serum samples. Moreover, the levels of Dihydrothy, Pantothenic acid, Aromadendrin 4'-methyl ether 7-rhamnoside were greatly increased in the AMI group in comparison with the control group. Nevertheless, LR obviously reduced the level of this metabolites. The results indicated that most of the metabolites were reversed by LR extract treatment and were regulated to return to levels that were similar to those of the control group.

9. Figure 8 belonged to speculation and/or hypothesis. Role of insulin or GLP-1 was not included. Why?

Response 9: Thanks very much for the reviewer's question and concern! The present study was aimed at exploring the hypoglycemic effect of LR in a type 2 diabetic rat model and examining potential biomarkers to obtain mechanistic insight into the serum metabolite modifications. Figure 8 is a schematic summary of metabolomic pathways and differentially expressed metabolites related to the LR effect in diabetic rats based on serum sample analyses. Our findings identified five metabolites as biomarkers that are involved in the regulation of amino acid metabolism as well as participate in acetyl-CoA and TCA cycle modulation, which may aid in the management of diabetics and blood sugar control. In addition, the present study did not conduct a validation experiment on the potential molecular pathways and their association with insulin and GLP-1 secretion; therefore, we did not include the role of insulin or GLP-1 in this metabolomics summary graph. We will definitely consider further researching the association between insulin or GLP-1 secretion and potential metabolite pathways. Hope our explanation can be acceptable, thank you!

10. Quantification of swertiamarine, sweroside, hesperetin, coumarin, 1.7-dihydroxy-3, 8-dimethoxyl xanthone, and 1-hydroxy-2,3,5 tri-methoxanthone as the main constituents of LR is required.

Response 10: Thanks for the reviewer's advice! Quantification analyses of the main compounds were performed and added in the Result section as the reviewer's advice.

Table 1. Content of active compounds measured in the LR extract

Active Compound	Regression Equation	R2	Linear range, ug	LR extract (mg/g dry mater)
Swertimarin	Y=99.413X+6.3517	1	1.953-9.826	91.10
Swertiside	Y=198.81X+0.8476	1	0.431-2.154	6.09
Hesperetin	Y=437.89X+0.0685	1	0.119-1.002	7.65
Coumarin	Y=365.15X+0.0103	1	0.118-0.590	3.04
1.7-dihydroxy-3.8-dimethoxyxanthone	Y=214.16X+2.3704	0.9999	0.098-0.494	29.28
1-hydroxy-2,3,5 trimethoxanthone	Y=90.498X+7.1204	0.9999	2.026-10.305	3.70

Reviewer #2：

Traditional Mongolian medicine has been widely used in Mongolian areas and is favored by local people. The authors detected the main active ingredients of the Lomatogonium Rotatum(LR) extract by HPLC, and discussed Lomatogonium Rotatum Extract Alleviates Diabetes Mellitus in Rats Induced by High-fat High- sugar diet. The study provided a solid contribution, and the findings strongly support the inhibitory effect of LR on diabetes. But the authors need to add some quantitative results.

Response: Thanks very much for the reviewer's comments! We appreciate the time and effort that the reviewer dedicated to providing feedback on our manuscript and are grateful for the insightful comments on and valuable improvements to our paper. We are incorporated most suggestions made by the reviewer. Those changes are highlighted within the manuscript as marked in red. Please see below, in red, for a point-by-point response to the reviewer's comments and concerns.

1. Title："Serum metabolites" is a clinical indicator of "diabetes" and should not be listed together in the title. It is recommended to delete serum metabolites.

Response 1: Thanks for the reviewer's advice! We have deleted the term "serum metabolites" from the title as suggested (Line 1,2).

2. Methods ： 1) Authors should add the extraction method for Lomatogonium Rotatum

Response 2: Thanks for the reviewer's comment! We have added a section "2.2 preparation of LR extract" in M&M and edited as below:

Line 67-72: LR was collected from Xilinhaote grassland, Inner Mongolia, China. Five kilograms of LR was washed, dried and then crashed into powder. LR powder was extracted three times with 95% ethanol for 3 hours each time.

The extract was combined and concentrated under vacuum at 60℃ and then freeze-dried. Carboxymethylcellulose sodium salt (CMC-Na) solution was used to dissolve the LR extract and the LR suspension was gavaged to animals at 0.5g/kg, 2.5g/kg, and 5g/kg according to the previous report [16].

3. Methods：2) Authors should provide HPLC analysis method

Response 3: Thanks for the reviewer's advice! The HPLC analysis method was provided in M&M section 2.4 as below:

Line 96-101: HPLC analysis was performed on an Agilent 1260 InfinityII HPLC system. ZORBAX SB-C18 5-Micron column (4.6×250mm) with mobile phase water (A) -0.1% phosphate aqueous solution (B) and gradient elution (0-15 min, 30%-35%B; 15 to 25 min, 35% 50% B; 25 to 35 min, 50% 65% B; 35 to 45 min, 65% 70% B; 45 to 50 min, 70% 80% B; 50 to 55 min, 80% to 95%. 55-60min, 95%-100%); The flow rate was set to 1.0 mL·min-1, the column temperature was set to 30℃, and the detection wavelength was set at 234nm. Standards of six compounds were purchased (Sigma Aldrich) and calibration curves were performed.

4. Results：1) Authors need to provide changes in rat body weight.

Response 4: Thanks very much for the reviewer's suggestion! Changes in body weight data were provided in Figure 2A as below. The detailed description in Results section was as follows:

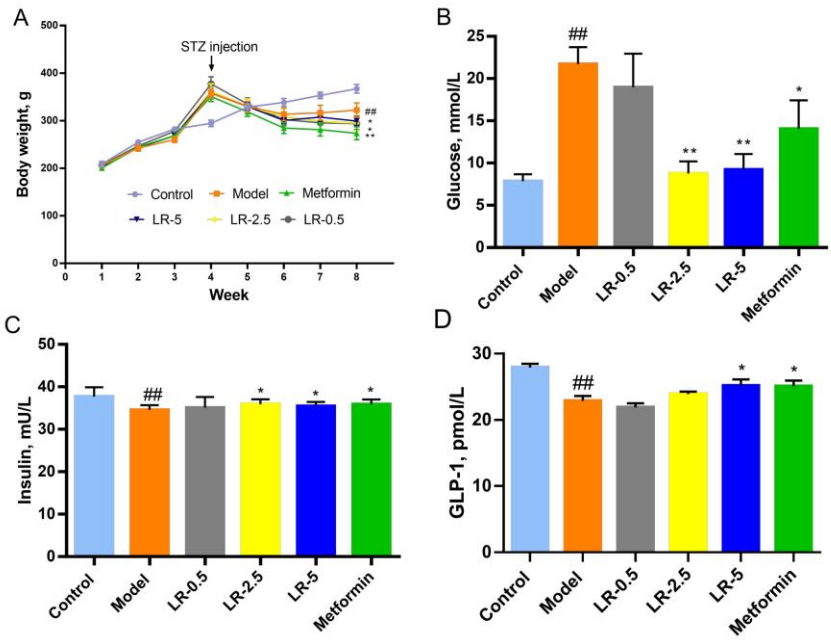


Figure 2. Effects of LR on Body weight (A), serum glucose (B), insulin (C), and GLP-1 (D) levels in the diabetic rat model. The data represent means±SEM (n = 10). ## $p < 0.01$ versus the control group; * $p < 0.05$ and ** $p < 0.01$ versus the model group.

Line 168-171: As shown in Figure 2A, the body weight was significantly increased in the HFHS diet-fed mice, while STZ injection sharply decreased the body weight in contrast to those in the control group. LR administration and metformin treatment groups significantly reduced ($P<0.05$ and $P<0.01$ respectively) the body weight of mice in comparison with those in the model group.

5. Results：2) It is recommended that the authors use the same format for drawings, such as Fig2, Fig3, and Fig5, and use the same color for the histogram.

Response 5: Thanks for the reviewer's advice! We have changed the format and color of Fig2, 3 and 5 as the reviewer's recommendation!

Figure 2

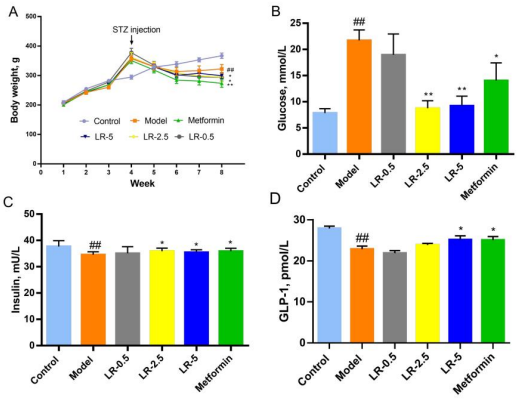


Figure 3

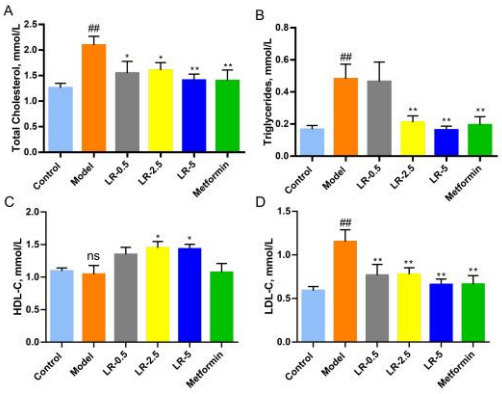
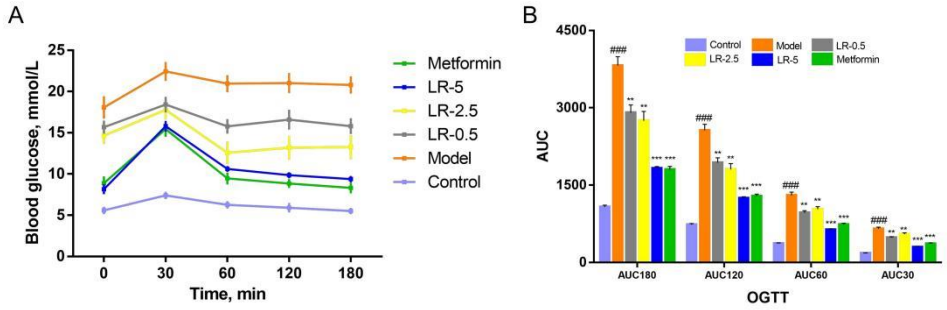
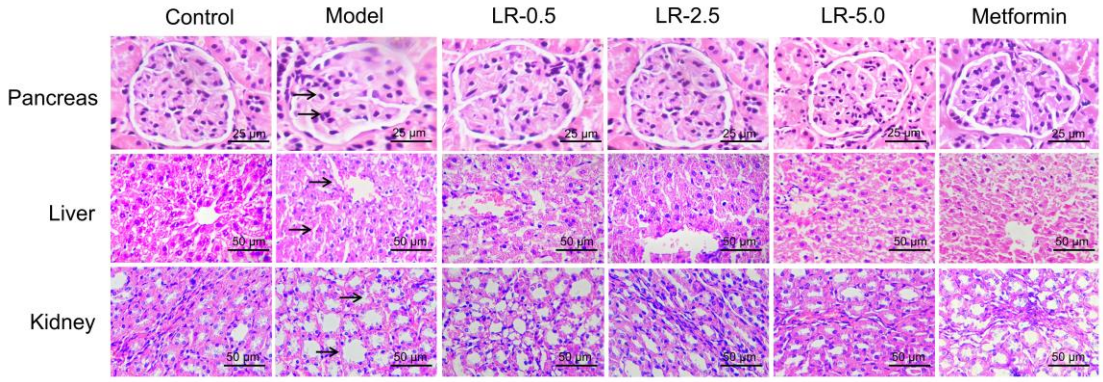


Figure 5



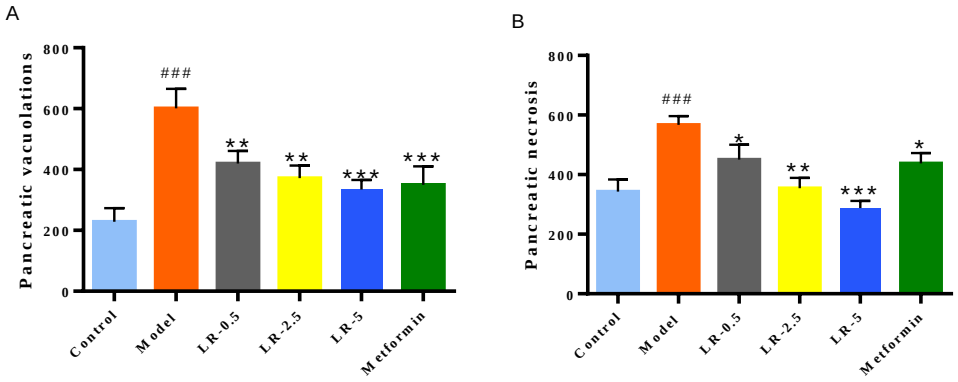
6. Results：3) The authors should provide clearer histological images with magnification or bar.

Response: Thanks for the reviewer's advice! We have changed several more clearer images and added the magnification bars as requested.



7. Results：4) The authors need to identify and quantify the extensive granulation of the β-cells and severe vacuolation of the pancreatic islets by ImageJ software or other software.

Response 7: Thanks very much for the reviewer's advice! The quantification of the β-cells granulation and islets vacuolation was performed and added as the supplementary figures. Thank you!



Supplementary Figure 1. Hematoxylin and Eosin (H&E) quantitative scoring of pancreatic vacuolation (A) and pancreatic necrosis (B).

8. **Discussions：1）Since LR is known for diabetes in clinical practice, surely it will produce a better effect in the degeneration of pancreatic islet β-cells of the model because it is clinically proven. Hence, the authors should prove which phytoconstituents of LR have a particular effect. This is very**

essential to prove in Response 8: Thanks very much for the reviewer's advice! Swertiamarin, hesperetin and coumarin, which have been previously reported with effects on obesity and hyperglycemia, making them the likely effectors of the pharmacological activities of the LR extract. The speculation and discussion were added in the Discussion section as below.

Line 285-290: According to an HPLC analysis, six main compounds were identified from the LR extract. The most abundant components are swertiamarin, hesperetin and coumarin, which have been previously documented with effects on obesity and hyperglycemia [20-22], making them the likely effectors of the pharmacological activities of the LR extract. The presence of xanthone, another key component in LR extract, is known to have numerous pharmacological effects, including antiinflammatory and antimycobacterial properties [23]. Nevertheless, its hypoglycemic action has yet to be investigated.

[20] Patel N, Tyagi RK, Tandel N, Garg NK, Soni N. The Molecular Targets of Swertiamarin and its Derivatives Confer Anti- Diabetic and Anti-Hyperlipidemic Effects. Curr Drug Targets. 2018;19(16):1958-1967. doi: 10.2174/1389450119666180406113428. PMID: 29623834.

[21] Yang H, Wang Y, Xu S, Ren J, Tang L, Gong J, Lin Y, Fang H, Su D. Hesperetin, a Promising Treatment Option for Diabetes and Related Complications: A Literature Review. J Agric Food Chem. 2022 Jul 20;70(28):8582-8592. doi: 10.1021/acs.jafc.2c03257. Epub 2022 Jul 8. PMID: 35801973.

[22] Pan Y, Liu T, Wang X, Sun J. Research progress of coumarins and their derivatives in the treatment of diabetes. J Enzyme Inhib Med Chem. 2022 Dec;37(1):616-628. doi: 10.1080/14756366.2021.2024526. PMID: 35067136; PMCID: PMC8788346.

[23] Elsaman T, Mohamed MS, Eltayib EM, Abdalla AE, Mohamed MA.

Xanthone: A Promising Antimycobacterial Scaffold. Med Chem. 2021;17(4):310-331. doi: 10.2174/1573406416666200619114124. PMID: 32560609.

9.Discussions：2）The authors performed a metabolomic analysis, which is an important set of findings. A more detailed and extensive discussion is highly warranted by the authors.

Response 9: Thanks for the reviewer's comments and suggestion! The findings from metabolomics analysis were comprehensively discussed following the reviewer's suggestion as below.

Line 312-358: Metabolomics is a high-throughput technology that have been widely used for identifying biomarkers, revealing metabolic pathways, and unraveling the mechanisms of metabolic diseases (29). In this study, untargeted metabolomics technology was used to analyze serum metabolites and the metabolic pathways of LR administration and to explore its mechanism of lowering blood glucose and anti-diabetic action. Our findings revealed that the metabolic pathway of vitamin B6 was the most influential factor, followed by terpenoid backbone biosynthesis, selenium amino acid, pyrimidine, arginine, and proline. Metabolites such as pyridoxal, mevalonic acid-5P, proline, lysine and taurine have been well reported on the regulation of T2DM, dislipidemia, inflammation and oxidative stress (30-32). In addition, LR administration promoted energy metabolisms, related to amino acid (AA). Recent studies reported AAs may be potentially important in the prevention of diabetes and diabetes-associated complications [33]. Protein and glucose metabolisms are strongly interconnected and consequently regulated at the metabolic and molecular levels. AAs relate to glucose metabolism via gluconeogenesis, which is a catabolic breakdown of AAs. In metabolomics studies, two important potential biomarkers, i.e., D-proline and L-lysine, were identified. Lysine supplements decreased diabetic complications linked with

T2DM in the diabetic rat models and in vitro [34,35]. Lysine is an essential amino acid that plays a major role in calcium absorption, building muscle protein, and the body's production of hormones, enzymes, and antibodies. Animal and human studies have shown that it has also demonstrated various beneficial effects in the treatment/prevention of diabetes and/or its complications. In diabetes-induced animal models, lysine has shown beneficial effects in lowering blood glucose as well as acting as an inhibitor of protein glycation [36]. Lysine is known to react with glucose, with the glycated amino acid being excreted in the urine, and it has been shown to markedly minimize the glucose response to dietary carbohydrates without the influence on insulin response [37]. And lysine could be catabolized to participate the energy metabolism. One mechanism involves the conversion of lysine to glutaryl-CoA, which is then converted to acetyl-CoA [38]. In the TCA cycle, lysine is metabolized to 2-ketoglutaric acid, which then forms succinate. Additionally, proline accelerates insulin secretion in both clonal β-cells and isolated mouse islets [39,40]. In the current study, the elevated level of insulin in the LR group could be influenced by the high proline level. Moreover, proline could be converted to glutamate and metabolized to pyruvate, which is a key metabolite joining the TCA cycle [41]. The pyruvate, metabolized to acetyl-CoA participates in the regulation of energy metabolism. Subsequently, the inappropriate glucogenic metabolism caused by the HFHS diet could be recovered by LR administration (Figure 8). In this view, the LR administration has potential to elevate lysine and proline levels may help with diabetes management and blood sugar control.

Vitamin B metabolism was modified after LR administration and the levels of pyridoxal, a key metabolite was restored in the LR groups. Vitamin B6 is an essential cofactor in various transamination, decarboxylation, glycogen hydrolysis, and synthesis pathways involving carbohydrate, sphingolipid, amino acid, heme, and neurotransmitter metabolism. The active form of vitamin B6, i.e., 5'-pyridoxine phosphate (PLP), is associated with

protecting cells from DNA damage. PLP acts as a coenzyme in about 160 enzymatic reactions, regulating the metabolism of glucose, lipids, amino acids, heme, DNA/RNA, and many neurotransmitters [42]. Furthermore, vitamin B supplementation's effect in preventing diabetic microvascular complications has long been the subject of study. Studies of vitamins B6 (pyridoxine, pyridoxine 50-phosphate) and high-dose vitamin B1 have shown that proteinuria can be inhibited in diabetic animal models [43]. In patients with T2DM and nephropathy, the combination of vitamin B1 (thiamine) and vitamin B6 (pyridoxine) significantly reduced the glycosylation of leukocyte nuclear DNA [44]. Addressing the vitamin B deficiency associated with diabetes that has been seen in experimental diabetes, particularly in tissues where vascular problems develop, may help to achieve the therapeutic advantage of vitamin B supplementation [45,46].

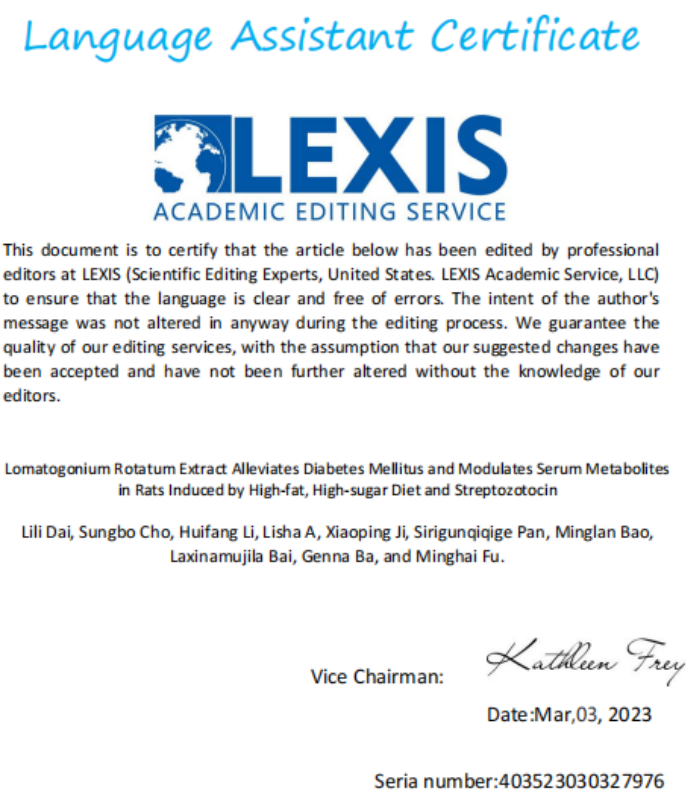
10. Discussions：3）Why author did not perform the toxicity study of LR to find out the effective dose? What basis is the therapeutic dose fixed? As per OECD guidelines, the toxicity study is very important before going for any pharmacological evolution.

Response 10: Thanks very much for the reviewer's question and concern! LR is a unique herbal medicine that has been extensively used in traditional Mongolian medicine clinics. It has been registered in the "Standard of Mongolian Medicine, Ministry of Health, China" and "Hospital Preparation of Mongolian Medicine, Inner Mongolia". In addition, the therapeutic doses of LR and its formulae were systematically tested and confirmed by a number of pharmacological and clinical studies. The most recent research progress of LR were updated and reviewed by our group (Dai LL, Eni RG, Fu MH, Ba GN. Botanical, chemical, and pharmacological characteristics of Lomatogonium rotatum: A review. World J Pharmacol 2022; 11(2)). Therefore, the therapeutic doses in the present study was referred to the LR clinical dosage and previous published reports. However, the scientific evidence of toxicity effect and

mechanism of LR are very important for evaluating its pharmacological (or clinical) efficacy and safety. We will definitely consider a further research into toxicity study of LR. Hope our explanation can be acceptable. Thank you!

11. English language should be revised (a mother tongue revision can be helpful)

Response 11: Thanks for the reviewer's suggestion! English language were carefully checked and edited by a professional English editing company LEXIS and provided the certificate.



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Lomatogonium Rotatum Extract Alleviates Diabetes Mellitus and Modulates Serum Metabolites in Rats Induced by High-fat, High-sugar Diet and Streptozotocin

Lili Dai, Sungbo Cho, Huifang Li, Lisha A, Xiaoping Ji, Sirigunqiqige Pan, Minglan Bao, Laxinamujila Bai, Genna Ba, and Minghai Fu.

Vice Chairman: Kathleen Frey

Date:Mar,03, 2023

Seria number:403523030327976

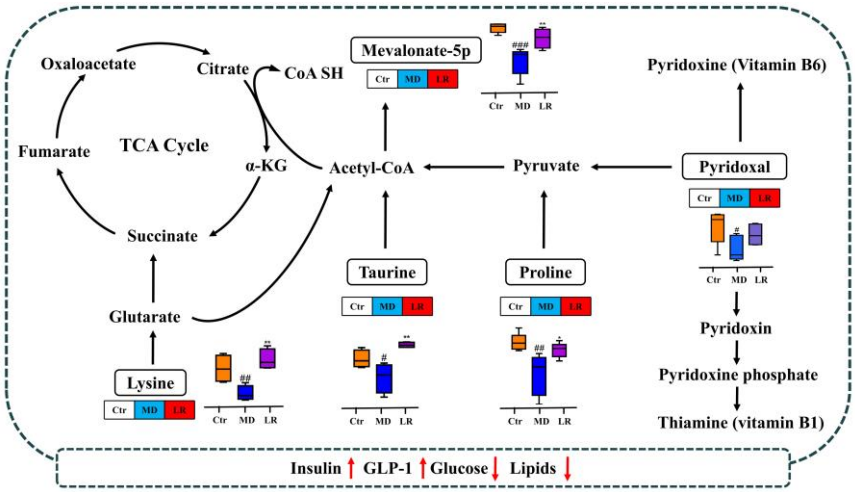
Revision reviewer：

1. It has been revised in a good way. One claim shown limitation(s) of current report may strengthen it in the further revise version because functional assay of GLP-1 was not included in current report. (Round 1 review comment: Figure 8 belonged to speculation and/or hypothesis. Role of insulin or GLP-1 was not included. Why?)

Response: Thanks very much for the reviewer's affirmative comment and a new suggestion on GLP-1 assay! By integrating the reviewer's both Round 1 and Round 2 comments, we have modified the Figure 8 that including the Insulin and GLP-1 in our speculative summary graph as below. The discussion and conclusion of GLP-1 can be found from line 306-311 and line 366-368 in the manuscript. Thank you!

Discussion (line 306-311) : Moreover, the plasma GLP-1 level was improved by the LR treatment. GLP-1 is a hormone primarily produced in the L-cells of the distal ileum and colon. It promotes insulin secretion while inhibiting glucagon synthesis. It also plays a significant role in glucose homeostasis and is a key biomarker of abnormalities in glucose metabolism [27]. The exposure of cultured gut endocrine cells to bitter substances stimulates the release of hormones, including GLP-1 [28]. Therefore, LR administration significantly improved insulin sensitivity and GLP-1 secretion in diabetic rats.

Conclusion (line 366-368): The results suggest that the hypoglycemic effect of LR may be associated with alterations in serum metabolites, which in turn may facilitate insulin and GLP-1 activities, leading to a reduction in blood glucose and lipid profiles.


Oxaloacetate
Citrate
CoA SH
Mevalonate-5p
Ctr MD LR
Pyridoxine (Vitamin B6)
Fumarate
TCA Cycle
α-KG
Acetyl-CoA
Pyruvate
Pyridoxal
Succinate
Taurine
Proline
Glutarate
Pyridoxin
Lysine
Pyridoxine phosphate
Thiamine (vitamin B1)
Insulin ↑ GLP-1 ↑ Glucose ↓ Lipids ↓