The subchronic toxic effects of *Mosla Chinensis Maxim* in normal rats.

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Research

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Abstract

Background: The aim of this work was to study the toxic effects and target organs of Mosla Chinensis Maxim (MCM) in rats and provide theoretical basis for clinical medication.

Methods: The subchronic toxicity study was conducted on 60 male and female SD rats using the fixed-dose method for the treatment group and 20 male and female SD rats for the control. At the subchronic toxicity study, the water extract of MCM with fixed-dose of 0.2g/kg/day, 2g/kg/day and 20g/kg/day was administered for 90 days intragastric, and the control group was given the same amount of distilled water. After 90 days, the general conditions of the rats were observed. Assesment on safety of the extract was conducted by a subchronic toxicity test which mainly examined alteration occured in gut flora and urine metabolism.

Results: The results showed that there were no significant toxic effects observed at all doses on physical sign and reactivity and fecal property of rats in the treatment groups had no obvious difference from those in control group. The results of routine blood test showed that the number of red blood cells in the male medium dose group and the female low dose group were significantly different from those in the control group ($P<0.05$). The results of serum biochemical indicators test showed that MCM had influence on the indicators of liver and kidney function, but it had no toxicological significance. In terms of glucose and lipid metabolism, the LDL level of male rats was lower than that of the control group ($P<0.05$). Compared with the control group, GLU level of female rats in the low, medium and high dose groups was significantly increased ($P<0.05$), indicating that long-term administration of MCM would affect the glucose level of female rats. The results of intestinal flora diversity showed that feeding MCM for 90 days had an impact on the distribution of intestinal flora. The content of lactobacillus increased and the ratio of Firmicutes and Bacteroidetes (F/B) was also affected, but there was no significant difference.

Conclusions: These findings showed that the long-term intragastric administration of the MCM is safe to use within its dose recommendation. But it could have slight affect the metabolism of uric acid by changing the composition of intestinal flora and affecting the metabolism of tryptophan.

Background

Mosla Chinensis Maxim (MCM)is the dry part of the Elsholtzia Maxim. Its nature: mild pungent, warm, into the lung, spleen and stomach, fatigue and discomfort, swelling, summer heat abundance and biological dam. The whole herb can be used as medicine and is clinically used to treat acute gastroenteritis, abdominal pain and diarrhea. MCM is traditionally used to cure Yin and summer heat caused by excessive indulgence in cool. Based on the research of the volatile oil chemical composition of MCM shows that its volatile oil has many pharmacological effects, such as pesticides, antioxidant, antibacterial, etc. (Qi et al. 2015). It is one of the components of Changyanning, a medicine for gastroenteritis (Mu-zi 2000). Due to its significant anti-inflammatory and other active effects, MCM has been listed as a tradition Chinese medicine with both medicinal and food functions. For scientific evaluation of the safety of MCM and mastering its security, in this study, the water extract of MCM was taken as the research object, through 90 days of gastric administration, the changes of intestinal flora were observed by using 16sDNA sequencing technology, and the changes of urine metabolism were observed by UHPLC-MS, in order to provide the basis for comprehensively understanding the safety evaluation of MCM.

Materials And Methods

2.1 Experimental Animals

The healthy SD rats were obtained from the experimental animal science and technology center of Jiangxi University of Chinese Traditional Medicine, of which half male and half female, 8 weeks old, and weighed 100 ± 10 g. The animal certificate number: SCXK (Gan) 2018-0003. Experimental rats were raised in SPF barrier system of experimental animal science and technology center of Jiangxi University of Traditional Chinese Medicine. In this experiment, a barrier housing facility was used in accordance with the national standard laboratory animal requirements of environment and housing facilities (GB 14925 – 2010). The rats were housed in acrylic cages lined with wood shavings at a constant room temperature (23 ± 1℃) and maintained on a 12 h light and 12 h dark cycle. The care of the laboratory animals and the animal experimental operation were performed in accordance with the committee of the Jiangxi University of Chinese Traditional Medicine (JZLLSC2019-0133).

Drug extraction

Experimental drug was purchased from Jiangzhong Chinese Traditional Medicine Decoction pieces Co., LTD. It was identified as the dry above-ground part of the labiform plant Elsholtzia MCM by Hao Chen, deputy director of pharmacy department of Affiliated Hospital of Jiangxi University of Traditional Chinese Medicine, and the decoction was prepared by the pharmacy department of the affiliated hospital, and the raw drug concentration of the decoction was 1.5 g/mL, batch number: 180823.

2.2 Experimental apparatus

Automatic blood coagulation analyzer (Japan Sysmex Corporation, version: Japan Sysmex, CA-1500); Automatic biochemical analyzer (American Beckman Coulter, version: Beckman Coulter AU480); Electric heating constant temperature water bath (Shanghai Yuejin Medical Optical Instrument Factory, version: HH.WB22-550-11); Ultra-pure water machine (Merck MILLIPORE, version: MILLI-Q-Integral 10); Inverted microscope (Japan Nikon, version: TS100-F); Electronic balance (Ihaus/Shanghai version: CP114); Multifunctional microplate reader (Thermofidel Technology Co., Ltd. Version: Varioskan Flash); High-speed refrigerated centrifuge (Shanghai Sai Murphysal Technology Co., Ltd., version: LYNX4000); Vortex oscillator (Haimen Qilin Bell Instrument Manufacturing Co., Ltd., version: Vortex KB3); UltiMate 3000 ultra-high performance liquid chromatography system, LTQ ORBITRAP VELOS PRO high-resolution mass spectrometer Instrument (Thermo Fisher Company).

2.3 Experimental reagents

ALT, AST, ALP, GGT, TB, DB, TP, ALB, CHE, TBA, UA, TG, TC, HDL, LDL, GLU, ADA, CRE, BUN level detection kit (Shanghai Kehua Biotechnology) Co., Ltd.), MDA, LPO, TNF, IL1, GAS, MTL, Na+-K+-ATP, CGRP, ET-1 level of blood detection kit (provided by Shanghai Hepai Biotechnology Co., Ltd.). HPLC grade (Methanol and acetonitrile) were purchased from Anaqua (Wilmington, DE USA). Distilled water was obtained from a Milli-Q Ultra pure water system (Millipore, Billerica, MA, USA).

2.4 Animal exposure

Eighty healthy SD rats, half male and half female, were randomly divided into eight groups, consisting of male/female normal control group (C), male/female low dose group (L), male/female medium dose group (M), male/female high dose group (H). Thus each group consisted of 20 rats. All rats were acclimatized for one week and given normal food and distilled water. The treatment was conducted according to the following procedures. In the normal control group, animals were administered normal food and equal amount of distilled water without

MCM water extraction, and the water extraction of the MCM at doses of 0.2, 2, and 20 g/kg/day for the low-dose group, Medium-dose group and high-dose group. The entire administration period was 90 days. It is administered once a day, and the oral dose is 1 ml/100 g body weight. The body weight of the rats was recorded once a week, and the dose of MCM was adjusted according to the new body weight. At the beginning of the experiment, the difference in animal weight did not over or under 10% of the average weight.

2.6 Observation indicators

2.6.1 Observation of clinical manifestations.

We observe the clinical manifestations of rats in the cage after gavage every day, including health status, feeding and drinking behaviors, food intake, and the color of the hair and fur.

2.6.2 Biochemistry analysis

At the end of the experiment, blood routine and liver and kidney biochemical function tests were done. Anesthetized with 2% pentobarbital sodium, and collected blood samples from femoral artery for hematology and blood biochemical examination.

2.6.3 Determination of immunology, gastrointestinal motility, antioxidation and energy metabolic capacity.

The levels of rat serum immunoglobulin (IgA, IgM, IgG) and complement (C3, C4) were measured by detection kits. The intestinal kinetics, oxidation and energy metabolism indexes of Na^+-K^+-ATP, MDA, LPO, TNF, IL-1, GAS, Mtl, CGRP, and ET-1 were measured by ELISA assay.

2.6.4 Histological examination

Dissected rats after blood collection, collected rat organs including heart, liver, spleen, lung, kidney, stomach, thymus, pancreas, adrenal gland, brain, male testis epididymis, female ovary, uterus and weighed them. Fixed and stored with 4% formaldehyde solution. All organs were washed and dehydrated, paraffin embedded, sectioned, stained with hematoxylin-eosin (HE) and observed under an optical microscope, and routine pathological examinations were performed.

2.6.5 Determination of intestinal flora

The experimental rats were fasted on the day before dissection, and the feces were collected from the anus by tail-lifting stimulation, placed in a cryotube and frozen at -80 °C, and the sequence examination of intestinal flora was conducted in the Shanghai Majorbio Bio-pharm Technology Co., Ltd.

2.6.6 Component identification of urine

UHPLC analysis was performed on a Ultimate 3000 UHPLC system (Thermo Scientific, San Jose CA, USA) and the chromatographic separation was carried out using ACQUITY UPLC BEH C18 1.7 μm Column (2.1 × 100 mm, Waters Corporation, Ireland). The UHPLC mobile phase consisted of 0.1% aqueous formic acid (solvent A) and acetonitrile (solvent B). The gradient duration was presented in Table S1. The column temperature was kept constant at 40 °C. A blank sample is applied between every ten samples to wash the column.

An orbitrap mass spectrometer (LTQ ORBITRAP VELOS PRO, Thermo Fisher Scientific, San Jose, CA, USA) equipped with a heated electrospray ionization (HESI) probe was coupled to the UHPLC system. Detection was performed in the negative ionization. The resolution type was set to 60000 FWHM and all samples were operated under the full scan mode. Dynamic exclusion was set to exclude a precursor ion for repested MS/MS analysis within 15 s. The activation type was collision induced dissociation (CID) and the intensity threshold was set at 1000. Other ion-source parameters of MS experiments were set as follows : Heater Temp, 350℃; Sheath Gas Flow Rate,35arb; Aux GAs FLow Rate, 10arb; Sweep Gas Flow Rate, 0arb; I Spray Voltage, 3.6 KV; Capillary Temp, 320 ℃; Mass range, m/z 100–1000.

2.7 Statistical analysis

The data was tested for significance between groups using IBM SPSS 26.0 application software, and single-factor ANOVA was used to test body weight, blood routine, blood biochemical indicators, and ELISA data. The UPLC-MS data were analyzed using SIMCA (Umetrics Company, USA) and also they were analyzed using principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) for multivariate statistical analysis. PCA, an unsupervised multivariate statistical approach, can reduce the dimensionalities of complex datasets and provide an overview of all observations in data tables. PLS-DA is used to reveal the net treatment effect on the subjects to detect the ions that have the greatest effect on the variance. Potential biomarkers were explored on the basis of variable importance in the project value (VIP > 1). VIP value of PLS-DA plays a major role in separation of the factors. The One-way analysis of variance (ANOVA) was applied to measure the significance of each metabolite. Metabolites with both multivariate statistical significance (VIP > 1) and univariate statistical significance ($P < 0.05$) were considered to be markers responsible for the differentiation of MCM treatment group from cont rol group. Finally, the ion spectrum of potential biomarkers was matched with the structure message of metabolites acquired from the Human Metabolome Database HMDB. Also please refer to the following databases: METLIN (http://metlin.scripps.edu/), KEGG (http://www.kegg.com/), and MZCloud (https://www.mzcloud.org/). GraphPad Prism 8.0.2 application software was for graphing software.

Results

3.1 Effects of MCM administration on clinical manifestations

The results showed that there were no significant physical toxicity signs and symptoms in behavior, activity, hair and fur color, food intake, drinking water, external reaction and feces between the experimental group and the normal control group.

3.2 Effects of MCM administration on body weight

As shown in Fig. 1, the hollow shapes represent female rats groups and solid shapes represent male rats groups. The body weight of all groups increased with the feeding time, and there was no obvious difference between the experimental groups and the normal control group ($P > 0.05$).

3.3 Effects of MCM administration on routine blood

After 90 days of administration, the blood routine test results of rats in each group were shown in Fig. 2. The numbers of red blood cells in the low and high dose female groups were significantly increased compared with the female control group ($P < 0.05$) (Fig. 2A), and the male rats in the middle dose group was significantly increased

compared with the male control group (Fig. 2B) ($P < 0.05$). No significant abnormalities were found in the number of other cells.

3.4 Effect of MCM on serum biochemical index

After the end of administration, serum was collected from each rat for liver and kidney biochemical index detection, and the results as shown in Fig. 3,4. The levels of ALT, GGT, ALP, TB, and DB of female rats in the experimental group were not significantly different from those in the control group. The AST level increased after administration, in all low and middle dose groups was significantly higher than that in the control group ($P < 0.05$) and with the increase of the dosage, there is a decreasing trend (Fig. 3a, 4a). The levels of TP, ALB and ADA in all experimental group were higher than those of control group, and there were a significant difference of TP and ALB levels compared with control group ($P < 0.05$).

The results of blood glucose and blood lipid parameters of female rats showed that there was no significant difference in LDL in the administration group compared with the control group, the levels of GLU, TG and TC were all higher than those in the control group, TG levels in the high-dose group and TC levels in the low-dose group were significantly different from those in the control group, HDL levels in the low-dose and middle-dose groups were significantly higher compared with the control group ($P < 0.05$), and the levels of GLU in the low-dose, middle-dose and high-dose groups were significantly different from those in the control group ($P < 0.05$), as shown in Fig. 3c. The levels of BUN and UA in the female administration group were no different from the control group, but the levels of CRE were significantly higher than those in the control group ($P < 0.05$), as shown in Fig. 3d.

3.5 Effect of MCM on energy metabolism, oxidation and gastrointestinal motility

The oxidation and energy metabolism indexes of rats were measured by ELISA assay, and the results were shown in Fig. 5 (female rats) and Fig. 6 (male rats). For energy metabolism, Na^+-K^+-ATP level in serum of all experimental groups was higher than control group and showed a certain dose-response relationship, but only the female experimental group showed a significant difference ($P < 0.05$). In terms of antioxidant capacity, MDA level of the administration group was not significantly different from that of the control group, but LPO level of the female high-dose group was significantly different from that of the control group ($P < 0.05$). For gastrointestinal motility, Mtl and ET-1 levels in all medium - and high-dose groups were significantly lower than those in the control group ($P < 0.05$), and CGRP levels in all male and female high-dose groups were significantly different from those in the control group ($P < 0.05$). For inflammation, IL-1 levels were significantly lower in all medium - and high-dose groups than in the control group

3.6 Effects of MCM on immunity

The results of serum immunoglobulin level were shown in Fig. 7. The levels of IgA, IgM, IgG, C3 and C4 did not show any difference ($p > 0:05$) compared to those in the control group.

3.7 Effects of MCM on routine urine

The results of routine urine test were shown in Table 1, and there was no significant difference between the experimental groups and the control group.

3.8 Effects of MCM on organ coefficient

As shown in Fig. 8, the organ coefficients of each administration group except the liver were not significantly different from the control group. Compared with the normal control group, the organ coefficient of the liver of female rats is slightly lower at medium and high doses (Fig. 8a). The liver organ coefficient of male rats increases with the increase of the administered dose, which has a significant dose escalation effect, and the high dose group has a significant difference ($P < 0.05$) (Fig. 8b).

3.9 Effects of MCM on histopatological

After 90 days, the rats were examined by routine autopsy, no obvious changes were observed by the naked eye. The rat liver, heart, spleen, lung, kidney, stomach, intestines, testis (came) and uterus (□) tissue for conventional histopathological examination, part organ pathological as shown in Fig. 9. No abnormal histopathological changes were found in the important organs under inverted microscope.

3.10 Effects of MCM on the Distribution of OTUs

We conducted 16S rDNA sequencing to profile gut microbiota composition and used a Venn diagram to show shared and unique OTUs number. For female rats, There were 527 common OTUs, 7 unique OTUs in the control group and 9,9,5 unique OTUs in the low, middle and high dose groups (Fig. 10A). For male rats, there were 557 common OTUs, While the unique OTUs in the control group were 7 and there were 5,12,12 unique OTUs in the low, middle and high dose groups (Fig. 10B).

As shown in Fig. 11A, MCM 90-day Feeding changes the level of intestinal flora, Females have greater influence on *Lactobacillus*, *Prevotella, Ruminococceaceae, Turicibaeter, Romboutsia*, and *Alloprevotella* relative Abundance. Compared with the control group, The relative abundance of *Romboutsia* and *Alloprevotella* are significantly different ($P < 0.05$), as shown in Fig. 11B. The differences between the sunburst map sets of multiple species at the bacterial genera level are shown in 11C.

The difference in intestinal microflora after 90 days feeding of MCM is shown in Fig. 12, and the results show that MCM can change the number of intestinal flora (Fig. 12A). In the female rat groups, Elsholtzia rugosa had a greater impact on *lactobacillus, prevotella_9, ruminococceaceae_ucg_014, turicibaeter, Romboutsia, Alloprevotella*, but the differences between the various genera did not reach a significant difference (Fig. 12B), the difference between the sunburst diagram groups of multi-level species at the genus level is shown in Fig. 12C.

3.11 Effects of MCM on urinary metabolism

The urine samples of the control and treated groups were analyzed by applying UHPLC - LTQ - Orbitrap - MS / MS in both positive and negative ionization modes to confirm the significant metabolic alterations of metabonomic profiles. The typical base peak intensity chromatogram obtained from the analysis of the positive ionization mode of the urine samples is shown in Fig. 12. The PCA plots for the treated groups groups clearly deviate from that of the control group by the 90th day (Fig. 13). The metabolic profiling had changed obviously, which led to further separation between the MCM treatment groups and control group.

The potential variables were selected as the biomarkers based on variable importance in projection values (VIP) value (> 1) and the ANOVA ($P < 0.05$). Finally, the 5 biomarkers (2 from the positive mode and 4 from the negative mode) were identified in urine (refer to Table 2) based on accurate mass, isotopic pattern, MS/MS information and comparison with the structure message of metabolites. As shown in table 3, Indoleacetic acid□N-Acetyl-L-

phenylalanine、2-Hydroxycinnamic acid、Kynurenic acid、Xanthurenic acid significantly decreased ($p < 0.05$). Furthermore, the urinary metabolites altered significantly in a dose-dependent manner. The 5 metabolites mentioned above demonstrated more significant changes in the high-dose group than that in the low-dose group. The metabolic profiling changed in high dose group appeared more remarkable effect than that in the low-dose group.

Discussion

Due to the long time and wide range of use of TCM, especially the dual use of TCM, the use of a single TCM may reflect the toxicity of TCM (Shuai-nan et al. 2015; Yiliang 2016) (Port 2019). Modern pharmacological studies have found that some herbal ingredients of aristolochliaceae are toxic (Wei 2017). For example, aristolochliic acid in them shows relatively strong toxicity and even irreversible permanent carcinogenicity. In recent years, more and more attention has been paid to the evaluation of the safety of Traditional Chinese medicine (Zhang Guangping 2016; Jiang-cun et al. 2016) (Xiaohe 2019; Jiabo, Haibo, and Keyong 2018) and there are many new methods being explored, such as network pharmacology(Gui and Jie 2012), genomics (Xue-ping et al. 2015), comparative omics and metabonomics (Zhuo et al. 2014) (Li-hua et al. 2015) (Dan-dan et al. 2016) (Nai-xi et al. 2012). TCM safety evaluation research based on TCM syndrome classification theory(Hui 2008), evidence-based pharmacy(Li Ling 2016), data mining (Yuan-yuan et al. 2011), etc. Mammalian 90-day toxicity test is an important experiment for safety evaluation, and the traditional 90-day feeding test has relatively few endpoints, narrow coverage and. Nowadays, some toxicologists began to explore the use of invertebrates as an alternative of mammals for the long-term studies (Hong et al. 2020). We added intestinal microbial diversity detection and urine mass spectrometry to the traditional 90-day feeding test analysis in order to obtain more comprehensive data for safety evaluation of subjects.

The weight of experimental animals during their growth and development is one of the most basic and sensitive indicators to comprehensively reflect the health status of animals(Zhong-ren et al. 2011), so it is an important observation indicator in the toxicity test. As well as the balance of lipid metabolism. In the 90-day subchronic toxicity study of Elsholtzia Maxim, it was shown that it had no significant influence on the weight and growth of rats. However, the weight of male rats from the second week showed that the weight of the administration group was slightly lower than that of the normal control group and the TC, LDL levels of the male administration group were significantly lower than those of the normal control group, the HDL level increased. The results are in line with previous studies that MCM has the effect of lowering blood lipids (lijuan 2011).

The blood routine index is an important index to reflect whether the test substance has affected the hematopoietic function. The results of this experiment showed that the number of red blood cells in female rats with low and high dose and male rats with medium dose increased after 90 days, but no obvious dose-effect relationship was observed, so comprehensive analysis showed that feeding experiment of MCM for 90 days had no obvious influence on blood routine of rats.

In order to improve the overall understanding of the safety of traditional Chinese medicine, we increased the detection of serum energy metabolism, oxidation index and gastrointestinal motility index. Motilin (Mtl) is one of the hormones of the digestive system, its function is to increase the migrating motor complex (MMC), promote gastrointestinal movement and stimulate the secretion of pepsin, while promoting gastric emptying(Haba 1993; Melmed et al. 2015). The results of this experiment showed that Mtl levels in all the treatment groups were decreased, and the Mtl levels in the medium and high dose groups were significantly lower than those in the

control group. Calcitonin gene-related peptide (CGRP) is a neuropeptide that is distributed in the brain and gastrointestinal system and has a variety of biological effects, it can protect gastrointestinal function by inhibiting gastric acid secretion, increasing gastric mucosal blood flow, slowing gastrointestinal movement, inhibiting inflammatory reaction, antagonizing free radical damage and regulating gastrointestinal hormone secretion. In this experiment, it was found that the CGRP level of male rats in the low-dose, middle-dose and high-dose groups and female rats in the high-dose group increased, and there were significant differences with the control group. It was reported that the increase of CGRP level could reduce the release of inflammatory factors TNF and IL-1 (Xiao et al. 2019), therefore, the results show that MCM can inhibit the release of inflammatory cytokines, to play a important role in regulating local inflammation. The results are in line with the clinical treatment of acute gastroenteritis and abdominal pain vomiting and diarrhea by MCM, which may have good resistance to intestinal accumulation heat and enhance the characteristics of intestinal peristalsis.

The levels of ALT, AST, ADA and CHE can reflect the damage and severity of liver cells. The biochemical indicators of liver function such as TP, ALB and CHE can reflect the anabolic function of cell protein, this indicates a decrease in Alb level in serum liver injur (Ying et al. 2018; Xian-ping, Wei-yan, and Cai-yan 2017); The levels of TB, DB, associates can reflect the function of liver excretion and secretion and detoxification, ALP and GGT levels can reflect cholestasis. AST and ALT are very important enzymes in the metabolic process of the body. When liver cells are damaged, AST and ALT are released, increasing the levels of AST and ALT in serum. However, when the increase of these two indicators does not exceed twice, there is no toxicological significance. This study found that AST and ALT increased in both the female and male administration groups, and the liver organ coefficient was larger than that of the normal control group. It shows that MCM has effect on the liver, but it is not toxic, and the pathological section shows normal.

BUN, CRE and UA were the indicators of renal function. BUN is the end product of amino acid and protein metabolism of the body, and is the main component of non-protein in blood. When the kidney is injured, the excretion of BUN decreases, leading to an increase in the level of BUN in the serum. CRE is a kind of small molecule product produced by muscle metabolism in the body, which can be filtered and excreted by the kidney. CRE level is an important indicator to detect the detoxification ability of the kidney (Yuliang et al. 2019). In this experiment, it was found that the effects of 90 days feeding of water extract from MCM on renal function had no significant toxicological significance.

Rich in the amount and type of gut microbes not only helps digestion and absorption of nutrients, it also has a huge impact on the ADME process of exogenous chemicals, at the same time these metabolites can adversely affect the abundance and diversity of the gut microbes, and analyzing the distribution of intestinal flora is the innovation of this experiment. From the results (Fig. 10), the distribution of intestinal flora in the administration group and the control group has changed. Compared with the normal control group, the flora with a significantly higher relative abundance of the MCM administration groups are *Prevotella, Ruminococeaceae and Romboutsia* (Fig. 11–12). In terms of urine metabolomics, it was found that the level of uric acid in the urinary metabolites of the administration group was significantly different from that of the normal control group, the results are similar to previous studies. Previous studies have found that the intestinal flora is involved in the regulation of uric acid excretion. Short-chain fatty acids (such as butyrate) regulate the proliferation and repair of intestinal epithelial cells, change the number and distribution of uric acid transporters in the body, and affect the transport and excretion of uric acid. Lim et al. (Lim et al. 2014) conducted a horizontal study and analysis of fecal microbes in the general population of Korea, and a two-year longitudinal study and analysis of fecal microbes in identical twin

pairs, and confirmed that the level of human blood uric acid and the dominant intestinal type of intestinal flora It is closely related. Among them, the dominant intestinal type of *Prevotella* (the main decomposition products are butyric acid, succinic acid, lactic acid, etc.) has lower blood uric acid than the dominant intestinal type of Bacteroides. In 2016, Stern et al. (Stern et al. 2016) used broad-spectrum gene sequencing technology to compare and analyze the stools of 23 patients with kidney stones and 6 general population, and also found the correlation between hemorrhagic uric acid and the abundance of Prevotella and Bacteroides. In the same year, Guo et al. (Guo et al. 2016) studied the intestinal flora and metabolites and found that the type and quantity of intestinal flora in patients with gout and the decrease in the content of broken chain fatty acid butyric acid are associated with xanthine dehydrogenase and uric acid in the intestine. The decrease in the level of uric acid in the urine may be caused by changes in the distribution of intestinal flora.

N-acetyl-L-phenylalanine is the product of phenylalanine N-acetyltransferase in the phenylalanine metabolic pathway. Changes in the level of N-acetyl-L-phenylalanine indicate that exposure to MCM will affect the metabolism of phenylalanine.Indole acetic acid is the starting point and central compound of the tryptophan metabolism pathway. Tryptophan metabolism is closely related to the central nervous system and immune regulation, and plays an important role in maintaining the normal physiological functions of the central nervous system. Therefore, the change of indole acetic acid content suggests that MCM can interfere with the tryptophan metabolism of normal rats. Previous studies have shown that tryptophan and its metabolites are involved in the development of liver steatosis and steatohepatitis (Ritze et al. 2014). Changes in ALT and AST levels may be related to the metabolic pathways of tryptophan.

Conclusion

Combining the above detection indicators, it is shown that the tested drug Mosla Chinensis Maxim water extract is actually non-toxic and safe for clinical application. The results provide a scientific basis for the further application of MCM.

Declarations

Ethics approval and consent to participate All experiments were carried out in adherence with the guidelines of the Institutional Animal Care and Use Committee of China and were approved by the Animal Care and Research Committee of Jiangxi University of Traditional Chinese Medicine.

Consent for publication The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All participants agreed to publish.

Availability of data and materials The data used to support the findings of this study are available from the corresponding author upon request.

Competing interests The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Authors' contributions (I) Conception and design: Zhiyong Liu.,Shouming Li; (II) Administrative support: Longxue Li., Tao Hong.; (III) Provision of study materials: Dan Lei,. Li Liu, Kun Shu; (IV) Collection and assembly of data: Shenghong Huang, Ming Zhou Pingdong Cai; (V) Data analysis and interpretation: Zhouyang Gu, Dan Lei; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

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Abbreviations

MCM: Mosla Chinensis maxim

RBC: Red blood cell

WBC: White blood cell

HGB: Hemoglobin

PLT: Platelet

ALB: Albumin

TBA: Total bile acid

ALT: Alanine transaminase

TP: total protein

ALP: Alkaline phosphatase

ADA: Adenosine deaminase

AST: Aspartate aminotransferase

CHE: Cholinesterase

TB: Total bilirubin

DB: direct bilirubin

GGT: Glutamyl transferase

LDL: low-density lipoprotein

TG: Triglyceride

TC: Total cholesterol

HDL: high-density lipoprotein

GLU: Glucose

BUN: Blood urea nitrogen

CRE: Creatinine

UA: Uric acid

MDA: Malondialdehyde

LPO: Lipid peroxide

GAS: Gastrin

CGRP: Calcitonin gene-related peptide

Mtl: Motilin

ET-1: Endothelin1

TNF: Tumor necrosis factor

IL-1: Interleukin1

IgG: Immunoglobulin G

IgA: Immunoglobulin A

IgM: Immunoglobulin M

C4: Complement 4

C3: Complement 3

TCM: Traditional Chinese Medicine

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Tables

Table 1. Effect of MCM on urine routine in rats

	□				□			
Index	C	L	M	H	C	L	M	H
Occult Blood	2/10	2/10	0/10	0/10	2/10	2/10	1/10	1/10
Protein	1/10	1/10	0/10	0/10	1/10	1/10	0/10	1/10
Bilirubin	2/10	1/10	0/10	1/10	1/10	1/10	1/10	1/10
Urobilin-ogen	<16	<16	<16	<16	<16	<16	<16	<16
Ketone body	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10
Leukocy-te	0/10	0/10	0/10	0/10	0/10	0/10	0/10	0/10

C. Normal control group; L. Low dosed group, water extraction of MCM 0.2 g/kg/day; M. Medium-dose group, water extraction of MCM 2g/kg/day; H. high-dose group, water extraction of MCM 20g/kg/day; "*" compared with the control group, P<0.05; "**" Compared with the control group, P<0.01.

Table 2. Effects of MCM on toxic biomarkers in rats

Retention time (min)	Measured mass (Da)	Calculate mass (Da)	Error (Da)	Scan mode	Identity	Elemental composition	p-value	Fold change
12.67	176.0706	176.1918	0.1212	+	Indoleacetic acid	C10H9NO2	5.8×10^{-5}	2.769
10.26	208.097	208.2337	0.1367	+	N-Acetyl-L-phenylalanine	C11H13NO3	1.9×10^{-4}	3.252
7.55	162.0571	163.1510	1.0939	-	2-Hydroxycinnamic acid	C9H8O3	3.1×10^{-9}	4.282
5.21	188.0362	188.1605	0.1243	-	Kynurenic acid	C10H7NO3	1.2×10^{-9}	4.699
3.94	204.0312	204.1599	0.1287	-	Xanthurenic acid	C10H7NO4	2.2×10^{-8}	3.355

p-value: High-dose group compared with the control group at the 90^{th} day.

Fold change was calculated by dividing the mean of the peak intensity of each metabolite from high-dose group relative to the control group at the 90^{th} day.

Table 3 and Table S1 were not provided with this version of the manuscript.

Figures

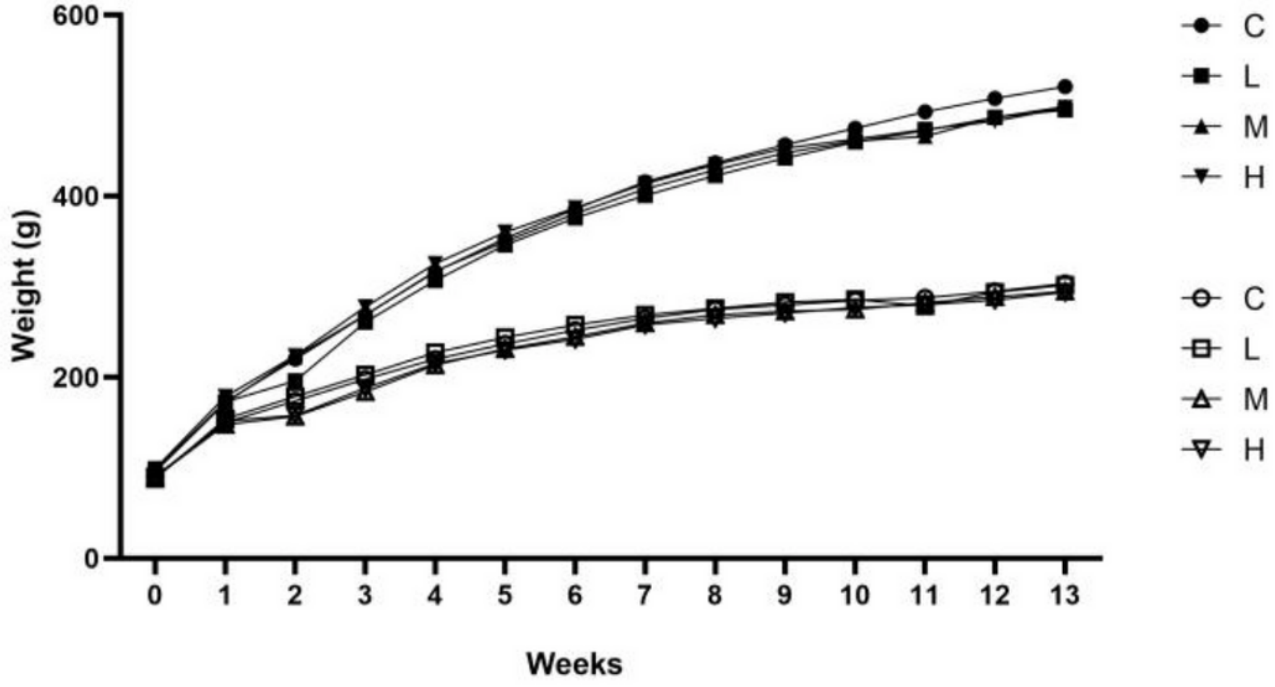

Figure 1

Effect of MCM on body weight in rats (n=10) C. Normal control group, L. Low dosed group, water extraction of MCM 0.2 g/kg/day M. Medium-dose group, water extraction of MCM 2g/kg/day H. high-dose group, water extraction of MCM 20g/kg/day

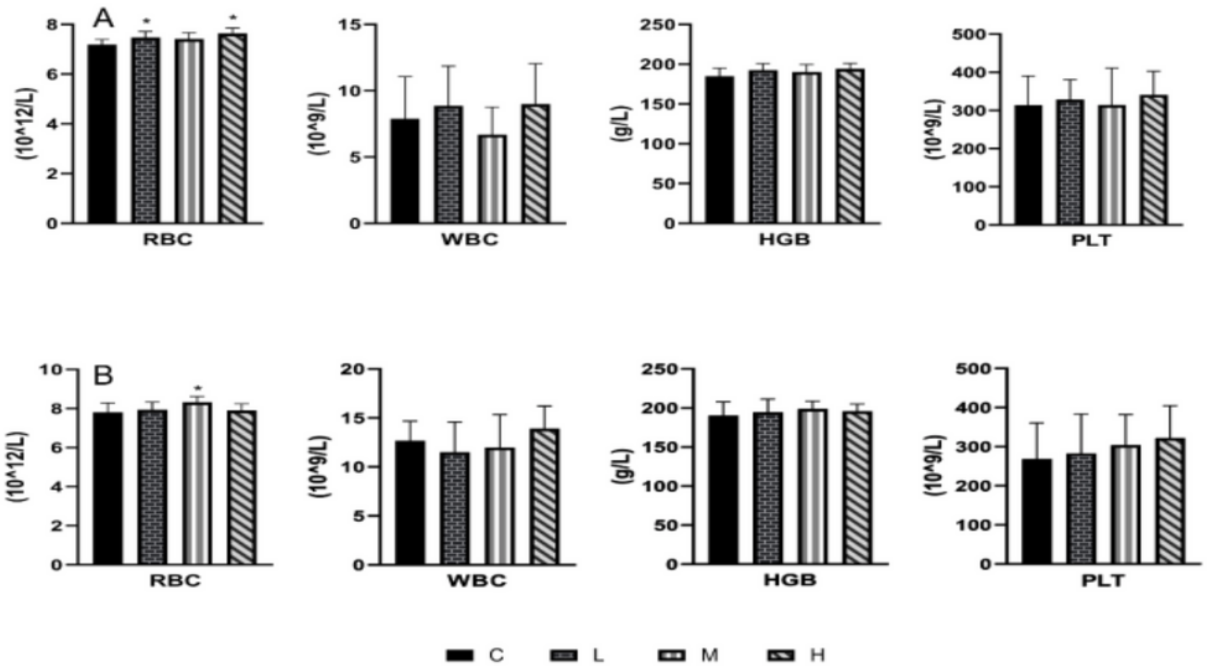

Figure 2

Effect of MCM on routine blood in rats C. Normal control group, L. Low dosed group, water extraction of MCM 0.2 g/kg/day. M. Medium-dose group, water extraction of MCM 2g/kg/day. H. high-dose group, water extraction of MCM 20g/kg/day."*" compared with the control group, P<0.05."**" Compared with the control group, P<0.01.

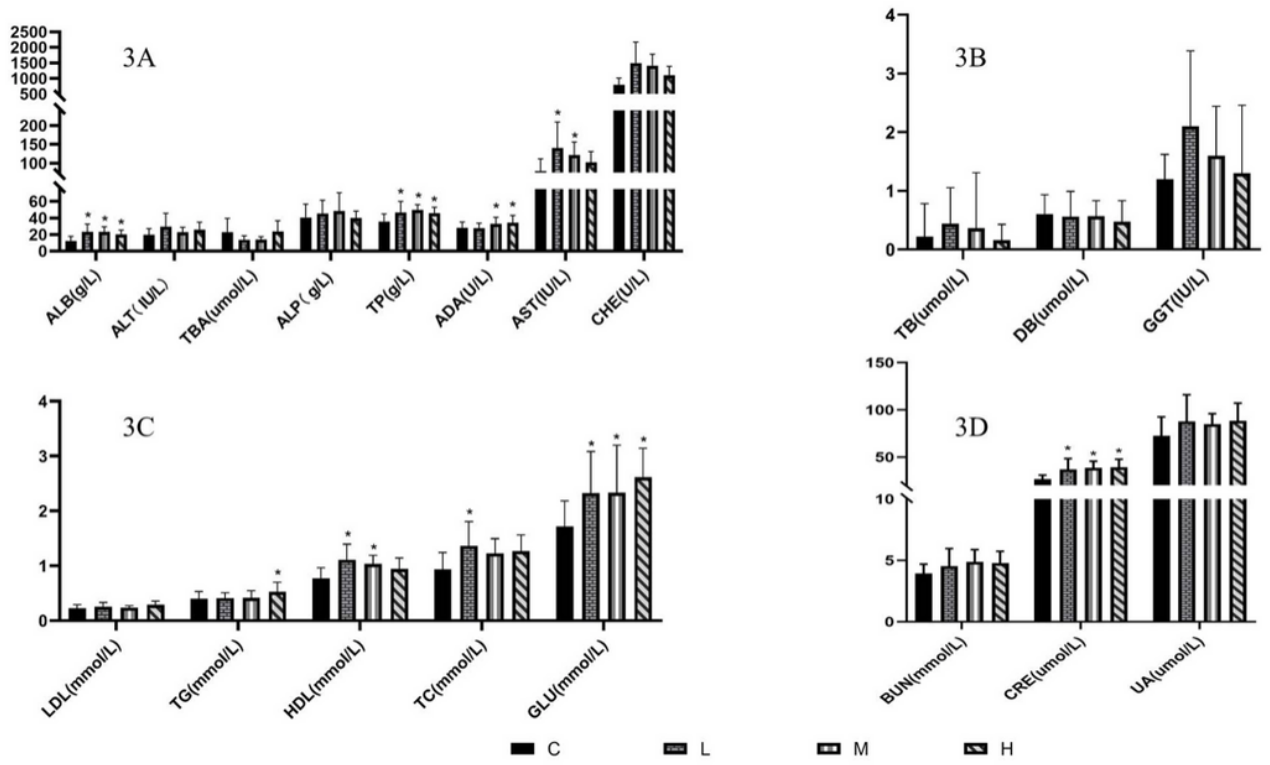

Figure 3

Effect of MCM on liver and kidney function related indexes, blood sugar and blood lipids in rats C. Normal control group.L. Low dosed group, water extraction of MCM 0.2 g/kg/day. M. Medium-dose group, water extraction of MCM 2g/kg/day. H. high-dose group, water extraction of MCM 20g/kg/day. "*" compared with the control group, $P<0.05$. "**" Compared with the control group, $P<0.01$.

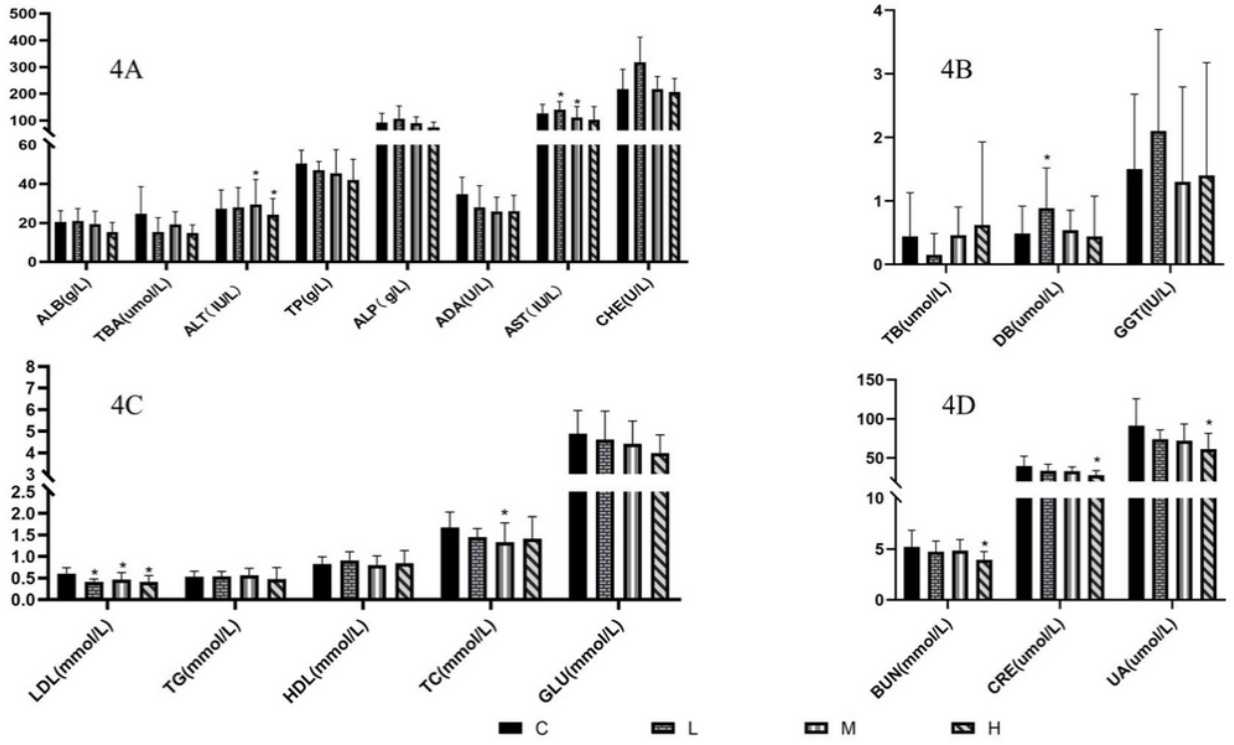

Figure 4

Effect of MCM on liver and kidney function related indexes, blood sugar and blood lipids in rats C. Normal control group.L. Low dosed group, water extraction of MCM 0.2 g/kg/day. M. Medium-dose group, water extraction of MCM 2g/kg/day. H. high-dose group, water extraction of MCM 20g/kg/day. "*" compared with the control group, $P<0.05$. "**" Compared with the control group, $P<0.01$.

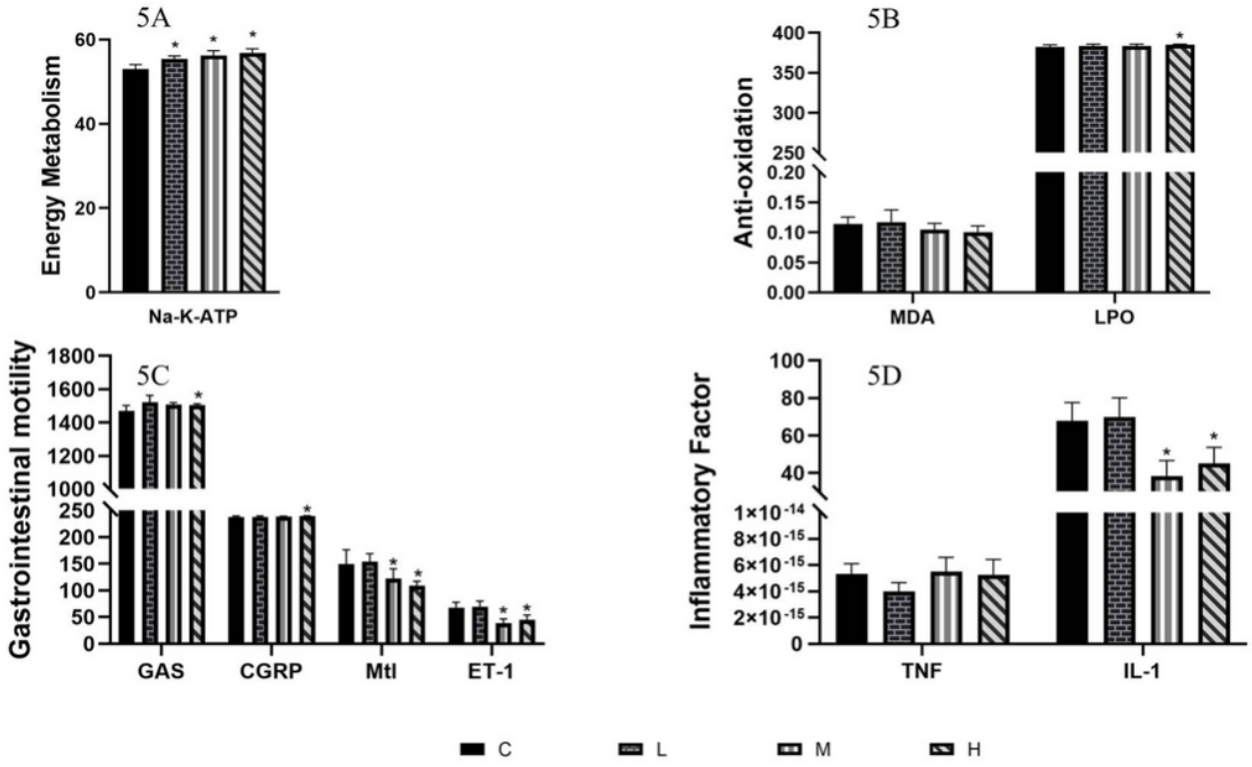

Figure 5

Effect of MCM on energy metabolism, oxidation and gastrointestinal motility in rats C. Normal control group. L. Low dosed group, water extraction of MCM 0.2 g/kg/day. M. Medium-dose group, water extraction of MCM 2g/kg/day. H. high-dose group, water extraction of MCM 20g/kg/day. "*" compared with the control group, $P<0.05$. "**" Compared with the control group, $P<0.01$

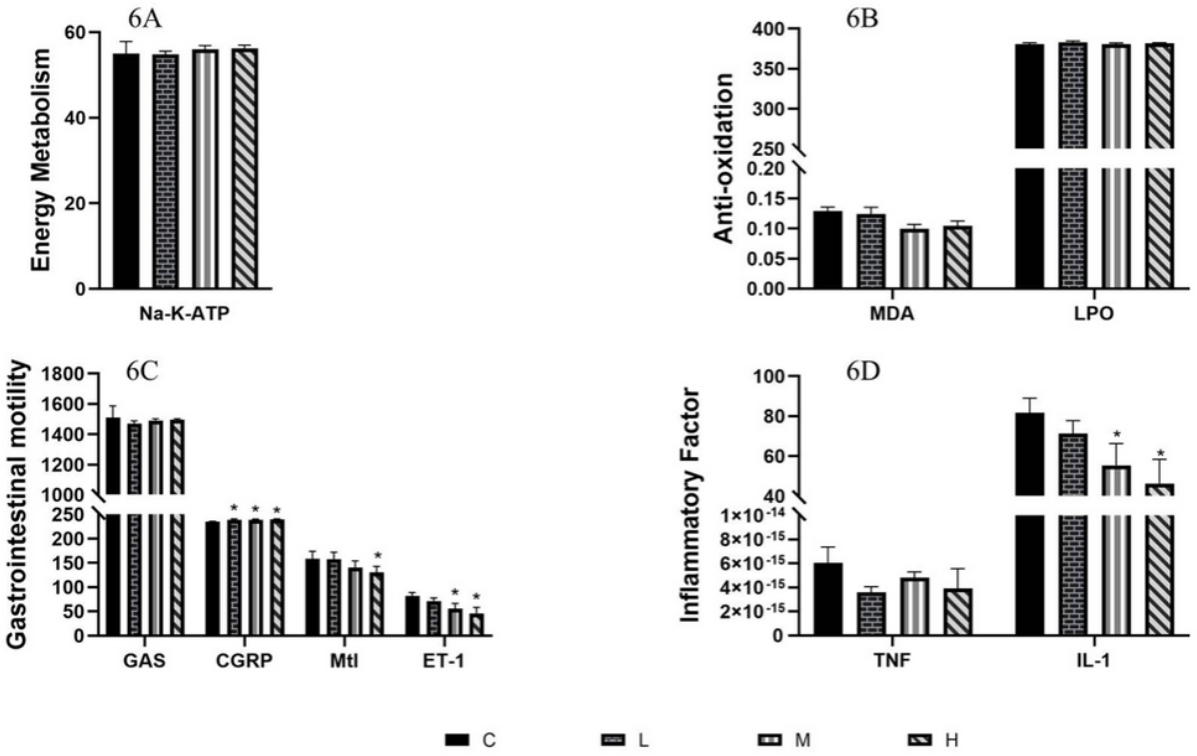

Figure 6

Effect of MCM on energy metabolism, oxidation and gastrointestinal motility in rats C. Normal control group. L. Low dosed group, water extraction of MCM 0.2 g/kg/day. M. Medium-dose group, water extraction of MCM 2g/kg/day. H. high-dose group, water extraction of MCM 20g/kg/day. "*" compared with the control group, $P<0.05$. "**" Compared with the control group, $P<0.01$

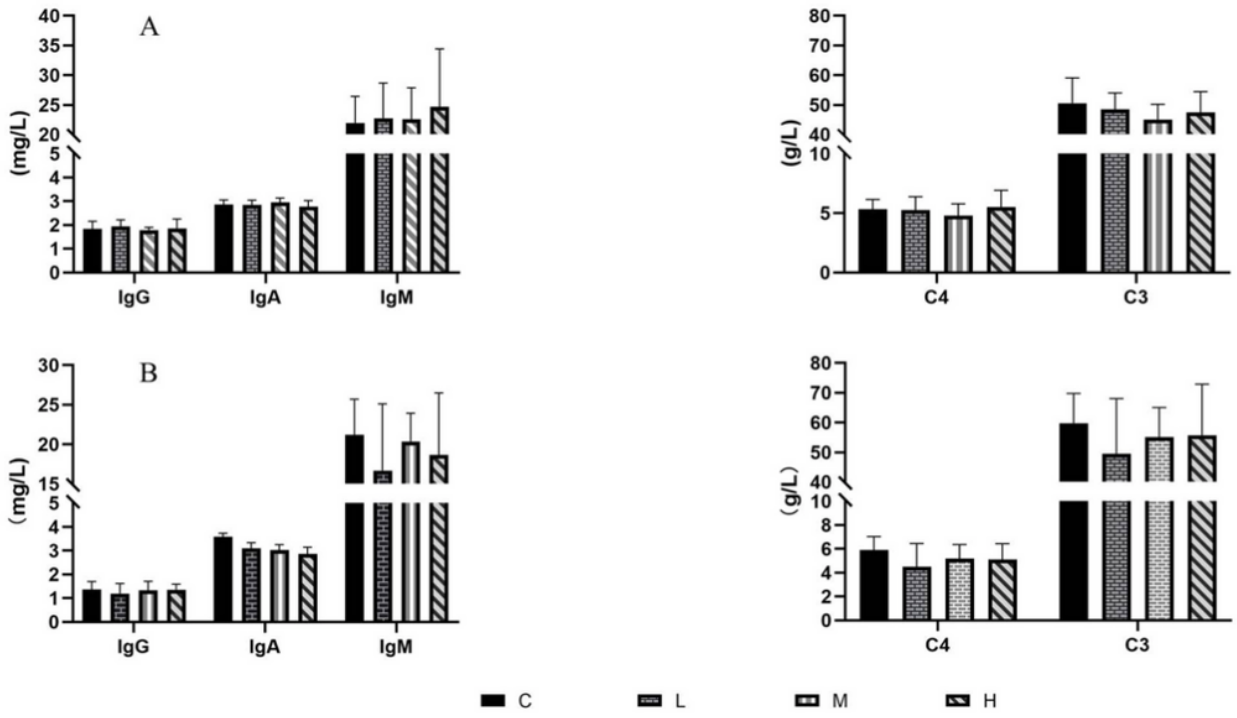

Figure 7

Effect of MCM on immunity in rats C. Normal control group. L. Low dosed group, water extraction of MCM 0.2 g/kg/day. M. Medium-dose group, water extraction of MCM 2g/kg/day. H. high-dose group, water extraction of MCM 20g/kg/day. "*" compared with the control group, P<0.05. "**" Compared with the control group, P<0.01

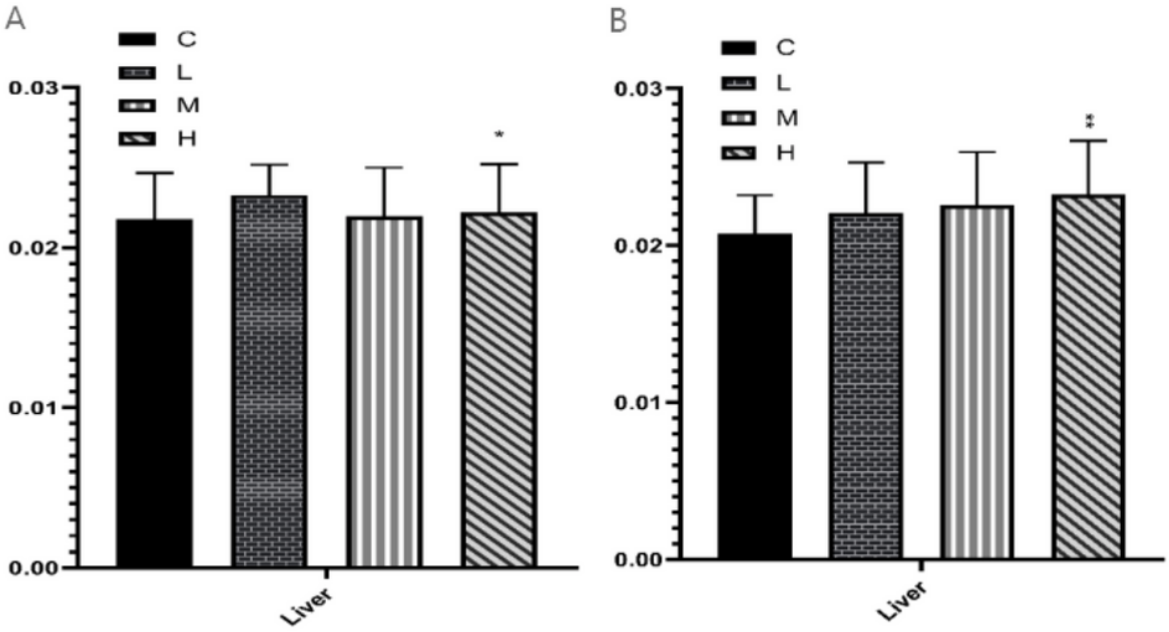

Figure 8

Effect of MCM on organ coefficient in rats (n=10). C. Normal control group. L. Low dosed group, water extraction of MCM 0.2 g/kg/day. M. Medium-dose group, water extraction of MCM 2g/kg/day. H. high-dose group, water extraction of MCM 20g/kg/day. "*" compared with the control group, $P<0.05$. "**" Compared with the control group, $P<0.01$

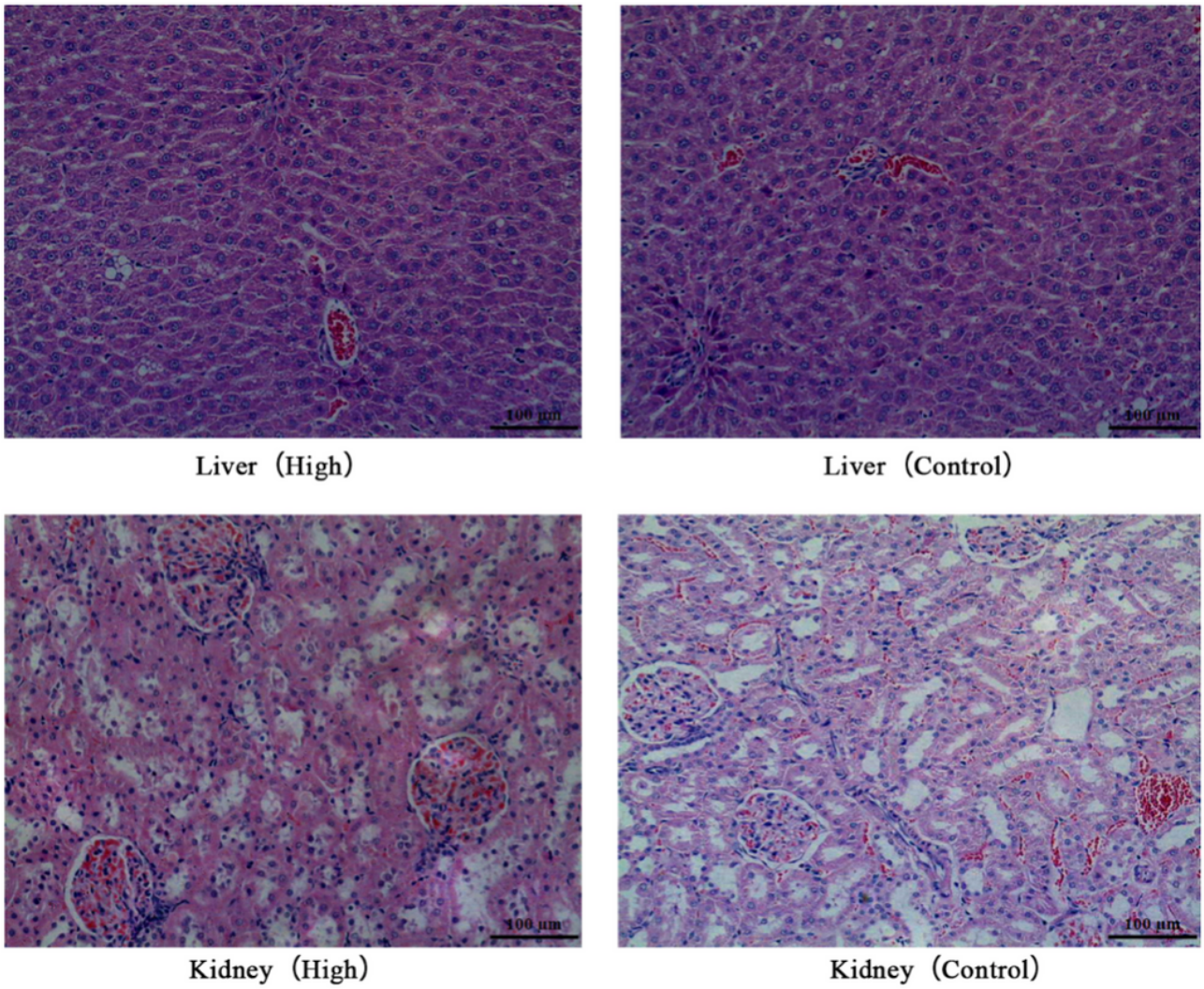

Figure 9

Effect of MCM on liver and kidney tissue pathology in rats

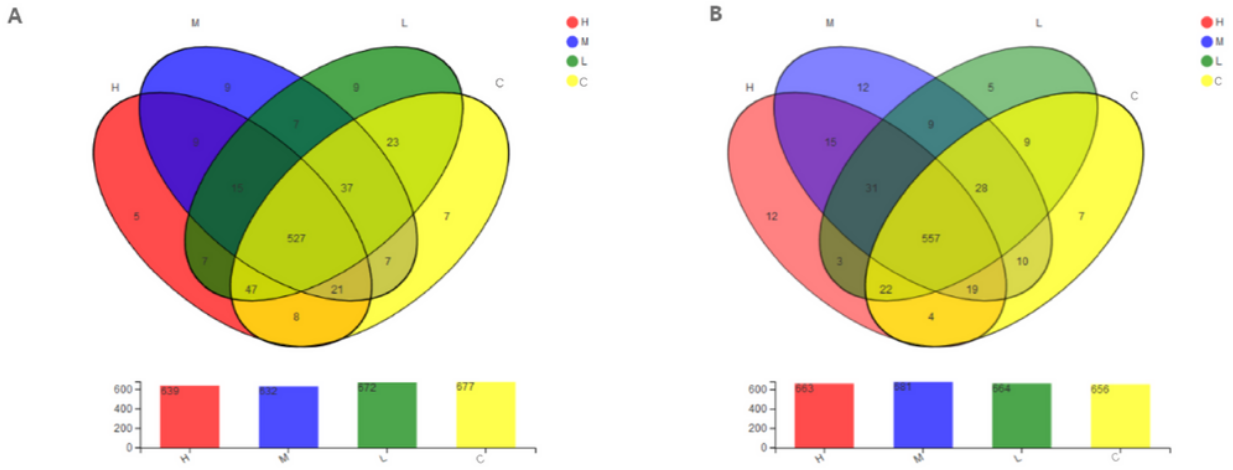

Figure 10

Effect of MCM on Common/unique OTU number distribution in rats C. Normal control group. L. Low dosed group, water extraction of MCM 0.2 g/kg/day. M. Medium-dose group, water extraction of MCM 2g/kg/day. H. high-dose group, water extraction of MCM 20g/kg/day.

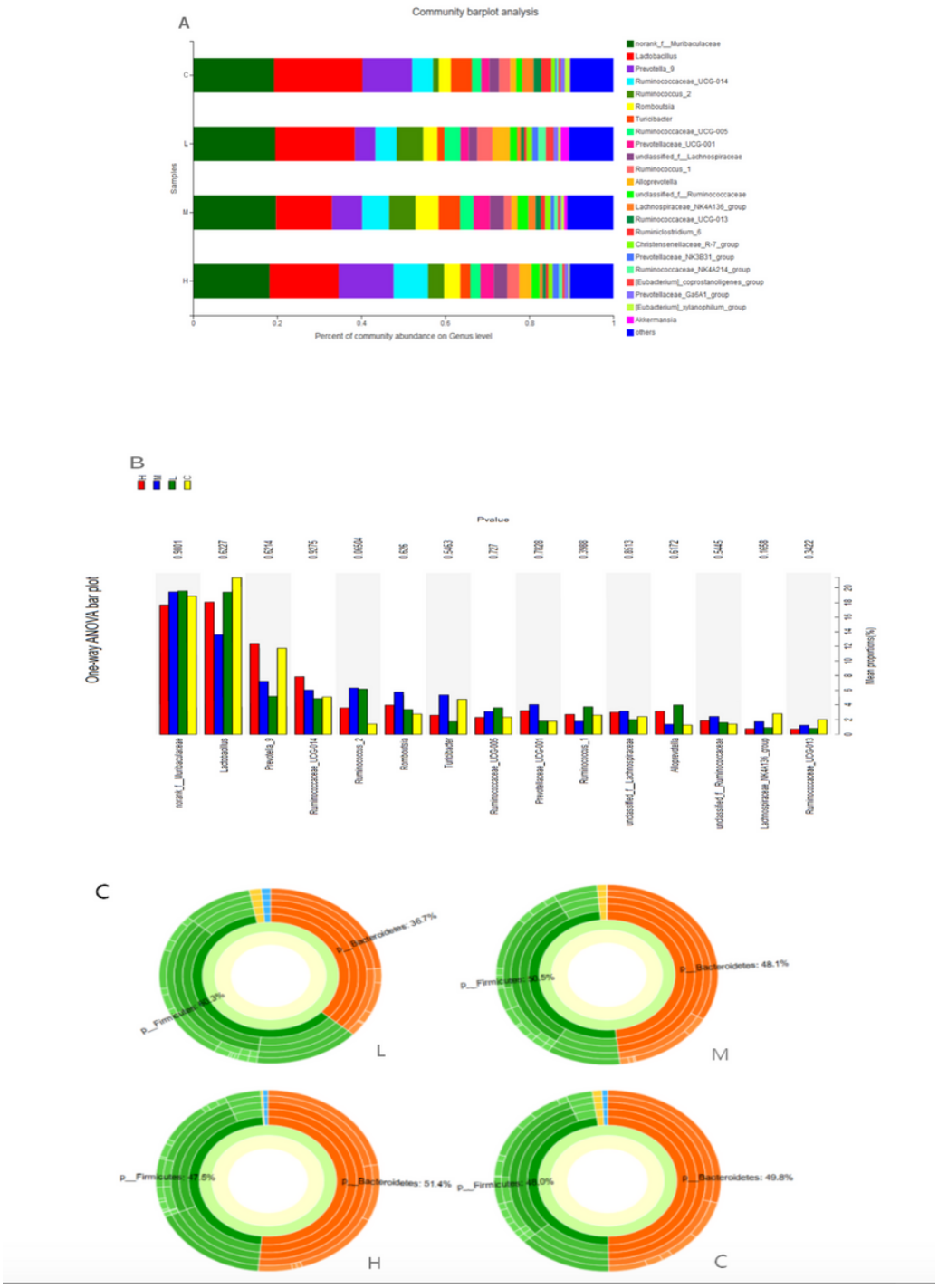

Figure 11

Effect of MCM on the relative abundance of gut microbiota in male rats C. Normal control group. L. Low dosed group, water extraction of MCM 0.2 g/kg/day. M. Medium-dose group, water extraction of MCM 2g/kg/day. H. high-dose group, water extraction of MCM 20g/kg/day. "*" compared with the control group, P<0.05."**" Compared with the control group, P<0.01.

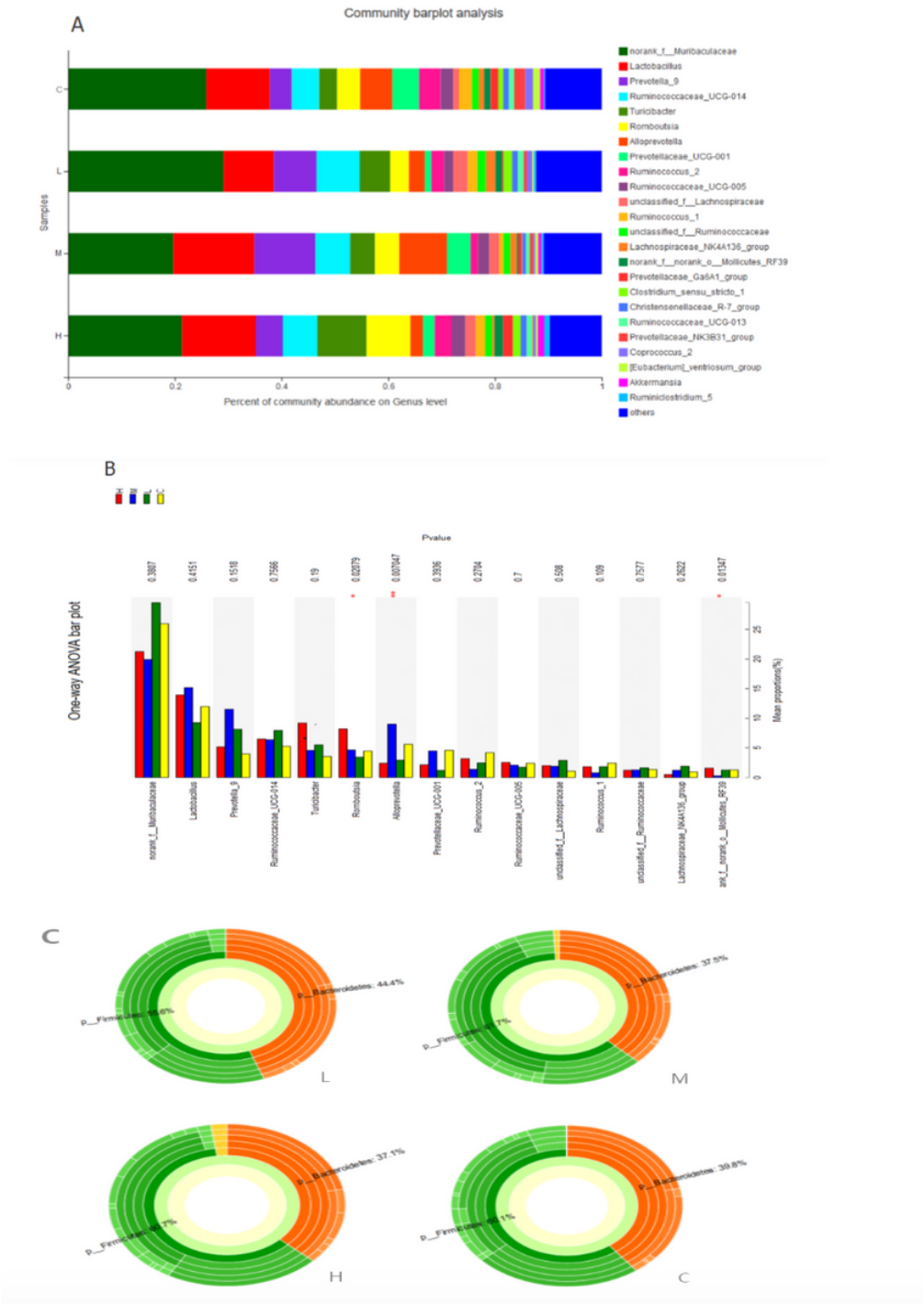

Figure 12

Effect of MCM on the relative abundance of gut microbiota in female rats C. Normal control group. L. Low dosed group, water extraction of MCM 0.2 g/kg/day. M. Medium-dose group, water extraction of MCM 2g/kg/day. H. high-dose group, water extraction of MCM 20g/kg/day. "*" compared with the control group, P<0.05."**" Compared with the control group, P<0.01.

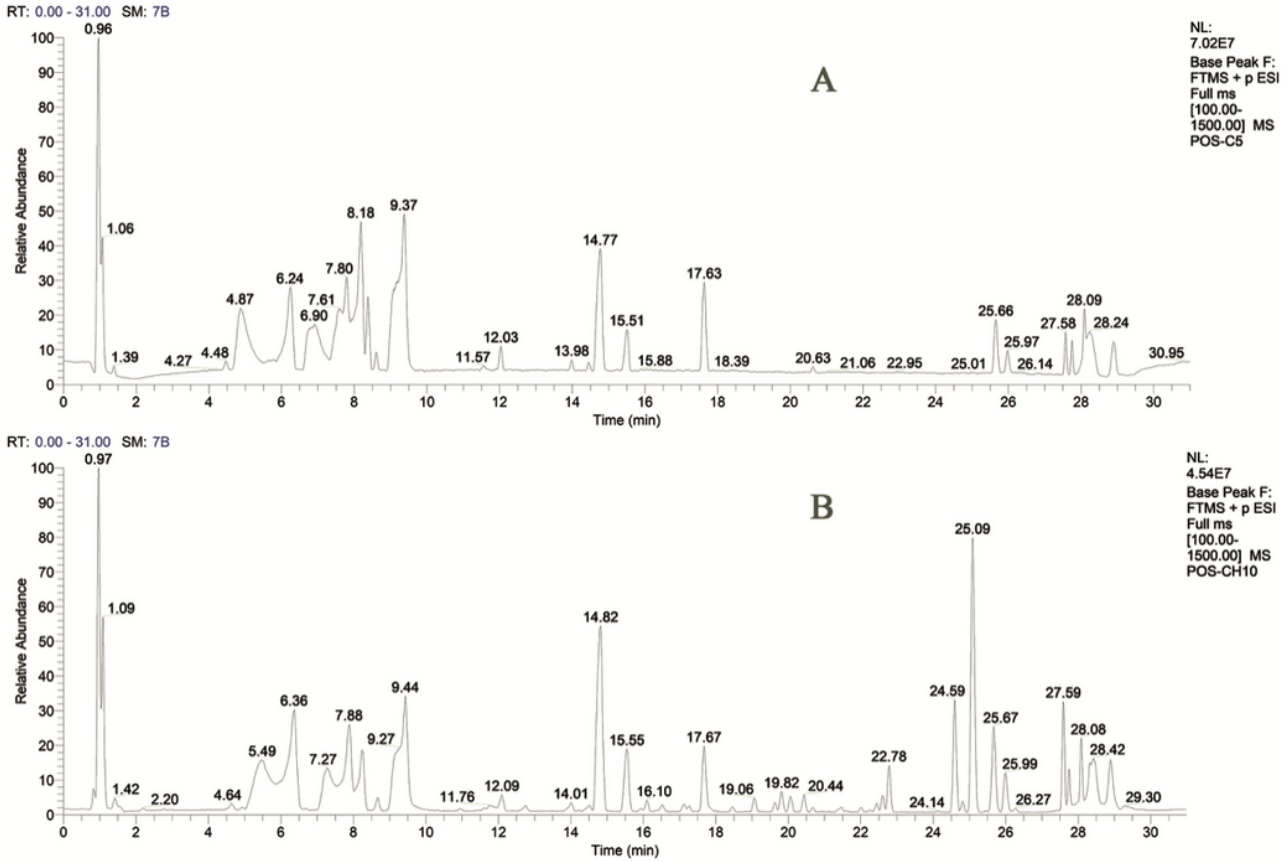

Figure 13

An orbitrap mass spectrometer equipped with a heated electrospray ionization(HESD) probe was coupled to the UHPLC system to intensity chromatogram of rat urine. (A)The positive ion basic peak intensity (BPI) chromatogram of the mixed sample of the control group and the experimental group (B) The negative ion basic peak intensity (BPI) chromatogram of the mixed sample of the control group and the experimental group

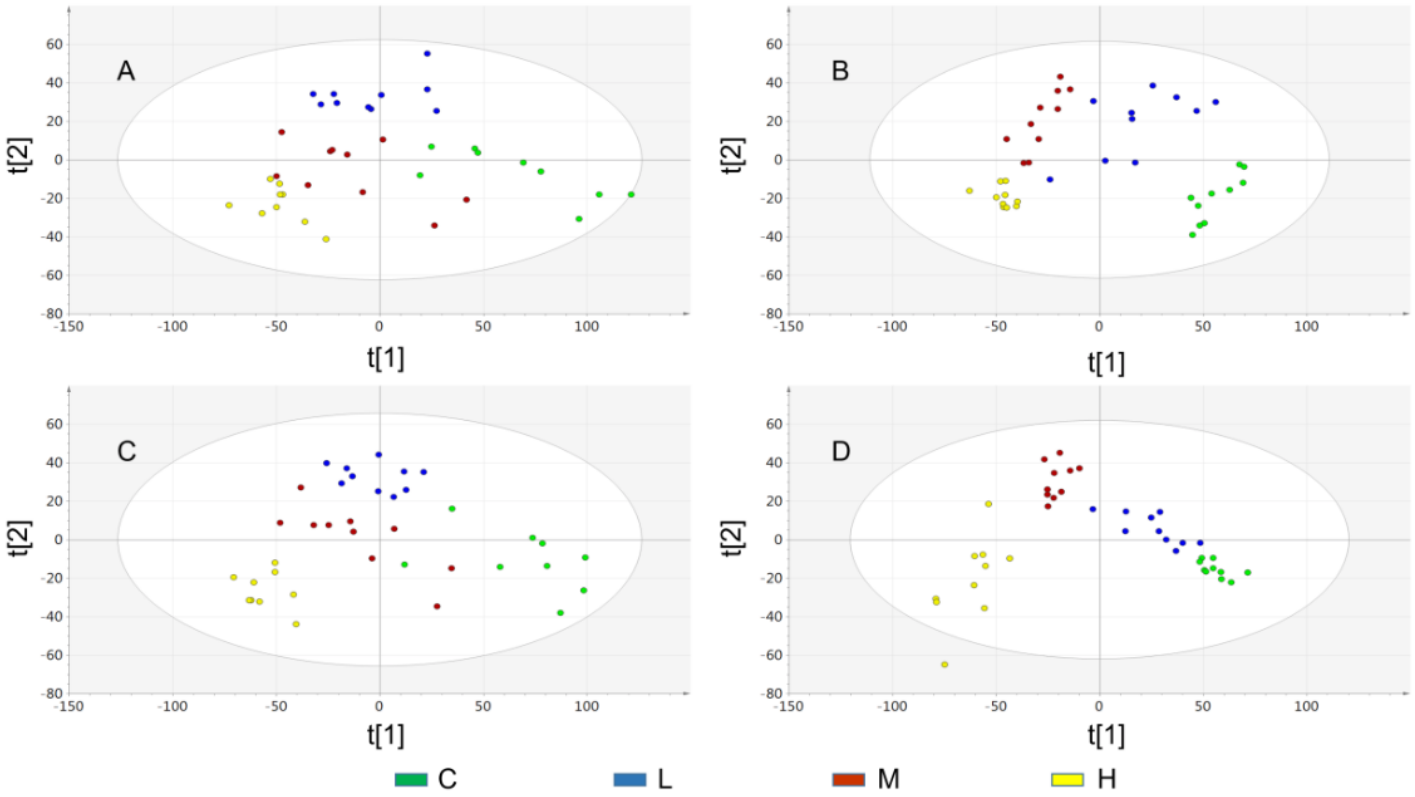

Figure 14

The mean PCA of the control and treated groups (A)-(B) in positive and (C)-(D) in negative. (A) 12 weeks after treatment in positive mode (R2X[1]=0.32, R2X[2]=0.0822); (B) 12 weeks after treatment in positive mode (R2X[1]=0.241, R2X[2]=0.0919); (C) 12 weeks after treatment in negative mode (R2X[1]=0.31, R2X[2]=0.091); (D) 12 weeks after treatment in negative mode (R2X[1]=0.273, R2X[2]=0.0837);