Letter to the Editor

Network Pharmacology and Experimental Study of Momordicine I and Momordicine II from Bitter Melon Saponins in Inhibiting Fat Accumulation*

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Bitter melon (*Momordica Charania* L.), a member of the Cucurbitaceae family, is widely distributed across tropical and subtropical regions. Saponin, an important functional component of bitter melon, has been proven to exert hypoglycemic effects similarly to insulin, and also possesses lipid-lowering properties inhibiting preadipocyte differentiation and fat synthesis[1]. As bitter melon saponin extract (BMSE) consists of compounds assembled in various diverse structures, it is necessary to conduct systematic and comprehensive research to screen its active ingredients and targets. In this study, we aimed to rapidly assess saponin compounds possessing lipid-lowering activity by combining LC/Q-TOF-MS/MS and network pharmacology. The bioactivity of screened saponins needs to be further verified *in vivo*, and the mechanisms underlying these behaviours must be assessed.

Caenorhabditis elegans (*C. elegans*), a form of nematode worms, is a species of eukaryotic animal with a short life cycle, and this species has attracted extensive attention for providing an intuitive way to evaluate fat accumulation, due to possessing homologous pathways of lipid metabolism with those of mammals. Accordingly, the intestinal tract of *C. elegans* is responsible for lipid uptake, synthesis, storage and mobilization. Oil Red O (ORO) staining and a triglyceride (TG) assay are typically applied to assess the influence of active compounds on lipid accumulation. One of our previous studies has shown that BMSE could significantly relieve fat deposition, both in *C. elegans* and in HepG2, possibly through lipophagy[2]. Lipid droplets (LDs) are multifunctional organelles present in eukaryotic cells that store triglycerides, cholesterol esters and other neutral lipids. The process of LDs being encapsulated by the autophagosome and hydrolyzed by lysosomal acid lipase (LAL) in the autolysosome is recognized as lipophagy. During lipophagy, adenosine 5‘-monophosphate-activated protein kinase (AMPK) and mammalian target of rapamycin (mTOR) connect the signaling axes of the autophagosome, Transcription Factor EB (TFEB) and Forkhead Box O1 (FoxO1) transcription factors, lysosome and autophagy associated proteins, where TFEB and FoxO1 directly induce the expression of LAL and promote β-oxidation. Whether the compounds we screened in BMSE can also decrease the accumulation of fat *via* lipophagy requires further investigation.

In the present study, we tried to screen potential active compounds with anti-obesity effects present in BMSE, through LC/Q-TOF-MS/MS and network pharmacology. Furthermore, we estimated the lipid-lowering effects of active compounds in *C. elegans,* and illustrated its potential mechanisms from the perspective of lipophagy, based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis.

BMSE was extracted from bitter melon juice using 80% ethanol (EtOH) and purified through use of AB-8 macroporous resin. The resulting concentration of BMSE was 9.26 mg/mL. The composition analysis of BMSE by LC/Q-TOF-MS/MS was followed by the determination of anti-obesity active components of saponins and their molecular mechanisms, using network pharmacology. Then, experimental studies were conducted in obese

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C. elegans, with the groups being divided into control, model, BMSE (25, 50, and 100 μg/mL), Momordicine I (25, 50, and 100 μg/mL), and Momordicine II (25, 50, and 100 μg/mL). We conducted growth index analysis, ORO staining, and TG assay to determine the lipid-reduction capacity of BMSE and its potential active ingredients in obese *C. elegans*. Furthermore, Monodansylcadaverine (MDC) fluorescent staining was applied to detect the formation of autophagosomes in *C. elegans*. The mRNA expression levels of *daf-16*/FoxO1, *hlh-30*/TFEB and lipophagy related genes of *C. elegans* were detected by quantitative real-time polymerase chain reaction (qRT-PCR). The primers used in this study are laid out in Supplementary Table S1 (available in www.besjournal.com). All data were analyzed as means ± SEM and were analyzed using the statistical package for the social sciences (SPSS 26.0) IBM Statistics 20 software (SPSS 26.0, Inc.). A one-way analysis of variance (ANOVA) using Tukey's range test was performed to identify the differences between independent sample groups. GraphPad prism 9.5 was used for graphing. Different letters present in the columns indicate statistically significant ($P < 0.05$) values.

Supplementary Table S2 (available in www.besjournal.com) shows the compounds identified *via* LC/Q-TOF-MS/MS. To screen for saponins with good bioavailability, 17 saponins were filtrated based on their Gastrointestinal absorption rate, including Momordicoside M, Momordicine II, Momordicine V, Momordicoside F2, and Momordicine I. A total of 73 common potential targets were identified by Venn analysis, out of 216 screened component-related targets (Supplementary Table S3, available in www.besjournal.com) and 1,125 obesity-related targets. In a protein-protein interaction (PPI) network, TNF, NTRK2, PPARG, MTOR, LRP1, ARC, F2R, JUND, TIMP1, CRHR1 were the top ten targets with high degree values (Supplementary Figure S1, Supplementary Tables S4–S5, available in www.besjournal.com). A recent report has shown that caffeine and quercetin could activate autophagy by inhibiting the mTOR signaling pathway, thereby reducing fat deposition in mice with obesity induced by high-fat diet[3]. Thus, mTOR were considered highly likely to play an important role in the anti-obesity benefit of BMSE.

To further elaborate on the biological functions and signaling pathways involved in the anti-obesity processes of BMSE, KEGG enrichment analysis was employed. The top 20 main targets were screened by *P*-value, including the AMPK signaling pathway, the insulin resistance, lipid and atherosclerosis, AMPK signaling pathway, PI3K-Akt signaling pathway and Type II diabetes mellitus (Figure 1A and Supplementary Table S6, available in www.besjournal.com). For the AMPK signaling pathway, a large amount of data showed that BMSE was a novel AMPK activator. Bitter melon derived triterpenoids could phosphorylate AMPK, and subsequently promote GLUT4 translocation to the cell membrane, thereby eliminating *in vivo* and *in vitro* hyperglycemia[4]. In addition, mTOR is a well-known key factor in the AMPK signaling pathway, regulating

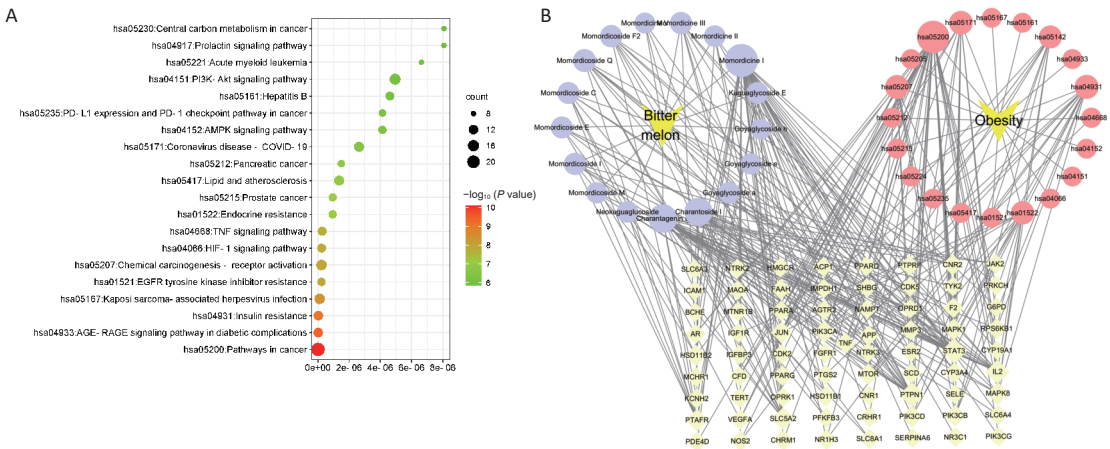

Figure 1. Screening the active saponins present in bitter melon saponin extract (BMSE) through Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis. (A) KEGG enrichment column of BMSE for potential targets of active ingredients associated with obesity. (B) Diagram of target pathway network of BMSE for the treatment of obesity. Values with different superscripts are significantly different ($P < 0.05$).

lipophagy by connecting autophagosome-TFEB and FoxO1 transcription factor-lysosome, in which TFEB and FoxO1 induce downstream targets including LAL, thereby promoting the β-oxidation of fatty acids[5]. However, there is little literature about how BMSE and its active compounds regulate lipophagy *in vivo* to inhibit fat accumulation.

Based on the betweenness value in the 'Compound-Target-disease-pathway' network, we observed that Momordicine I in BMSE was most likely to be the core component in treating obesity. Combined with the results of LC/Q-TOF-MS/MS, the relative content percentage of Momordicine I and II in BMSE was found to be 2.76% and 22.02% respectively. The response value of Momordicine II was relatively high, as Momordicine II may also regulate insulin-resistance and thus play a role in anti-obesity (Figure 1B and Supplementary Table S7, available in www.besjournal.com). Consequently, we selected Momordicine I and Momordicine II for further studies on lipid-lowering activity and its potential mechanisms, from the perspective of lipophagy based on the KEGG enriched pathways.

To verify the lipid-lowering effects of BMSE, Momordicine I and Momordicine II *in vivo*, an obese *C. elegans* model was established using high glucose. Supplementary Figure S2 (available in www.besjournal.com) shows that the concentrations of 25, 50, and 100 μg/mL of BMSE had no negative effects on the growth of nematodes. Lipids in *C. elegans* is mainly stored in the intestinal epithelial and intestine, in the form of TG. Compared with the model group, both BMSE, Momordicine I and Momordicine II were found to significantly reduce fat content in *C. elegans*, and the lipid-lowering effect was proportional to the dose concentration. 100 μg/mL Momordicine I degraded the fat content of *C. elegans* sharply in all treated groups ($P < 0.05$) (Figure 2). Lin et al. found that BMSE had a strong lipid-lowering benefit in both normal and high-fat *C. elegans*, as well as improved lifespan and healthspan. These data strongly confirm the lipid-reduction effects of BMSE and hint that Momordicine I played a leading role.

Based on the screening of anti-obesity targets of BMSE, mTOR is closely related to lipophagy with high degree values, and thus we speculated that BMSE may regulate lipid metabolism *via* lipophagy.

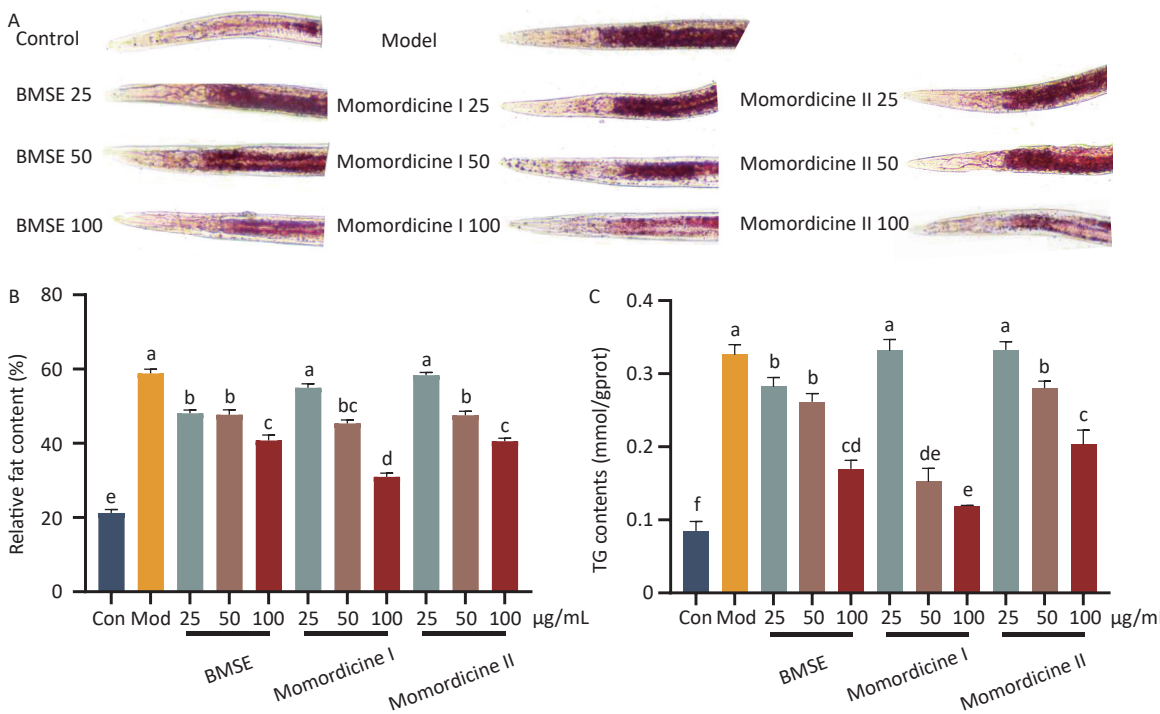

Figure 2. Effects of bitter melon saponin extract (BMSE) on lipid accumulation (overall fat and triglyceride content) of wild type (N2) *Caenorhabditis elegans* (*C. elegans*). (A) and (B) *C. elegans* were treated from L1 to L4 stage for 48 h followed by Oil Red O (ORO) staining. The Fat intensities were quantitatively analyzed by Image J from 15 to 20 *C. elegans.* (C) The triglyceride (TG) content was determined by kits. Results are shown as mean ± SEM. Values with different superscripts are significantly different ($P < 0.05$).

Lipophagy is a form of selective autophagy, catabolizing the components of LDs through LAL, which is a significant fat-degradation pathway besides lipohydrolysis. MDC fluorescent staining was applied to detect the formation of autophagosomes in nematodes (Supplementary Figure S3, available in www.besjournal.com). The green fluorescence was significantly enhanced (P < 0.05) in all treated groups, indicating that the number of autophagosomes remarkably increased *via* treatment with BMSE. By contrast, Momordicine I and Momordicine II had better effects than BMSE on the autophagy flow of obese nematodes, with Momordicine I exerting the best effect. Liao et al. found that dihydromyricetin clearly attenuated high FBS-induced inhibition of autophagosome formation in LO2 cells, similar to our results[6]. Therefore, we confirmed that BMSE alleviated lipid accumulation in nematodes *via* lipophagy. Nevertheless, its molecular mechanism required further investigation.

In order to study the mechanism by which BMSE and its constituent compounds regulate the lipid metabolism, the expression profile of lipophagy related genes was investigated by qRT-PCR (Figure 3A). The present study indicates that a high glucose diet inhibited *hlh-30* (TFEB homolog) gene

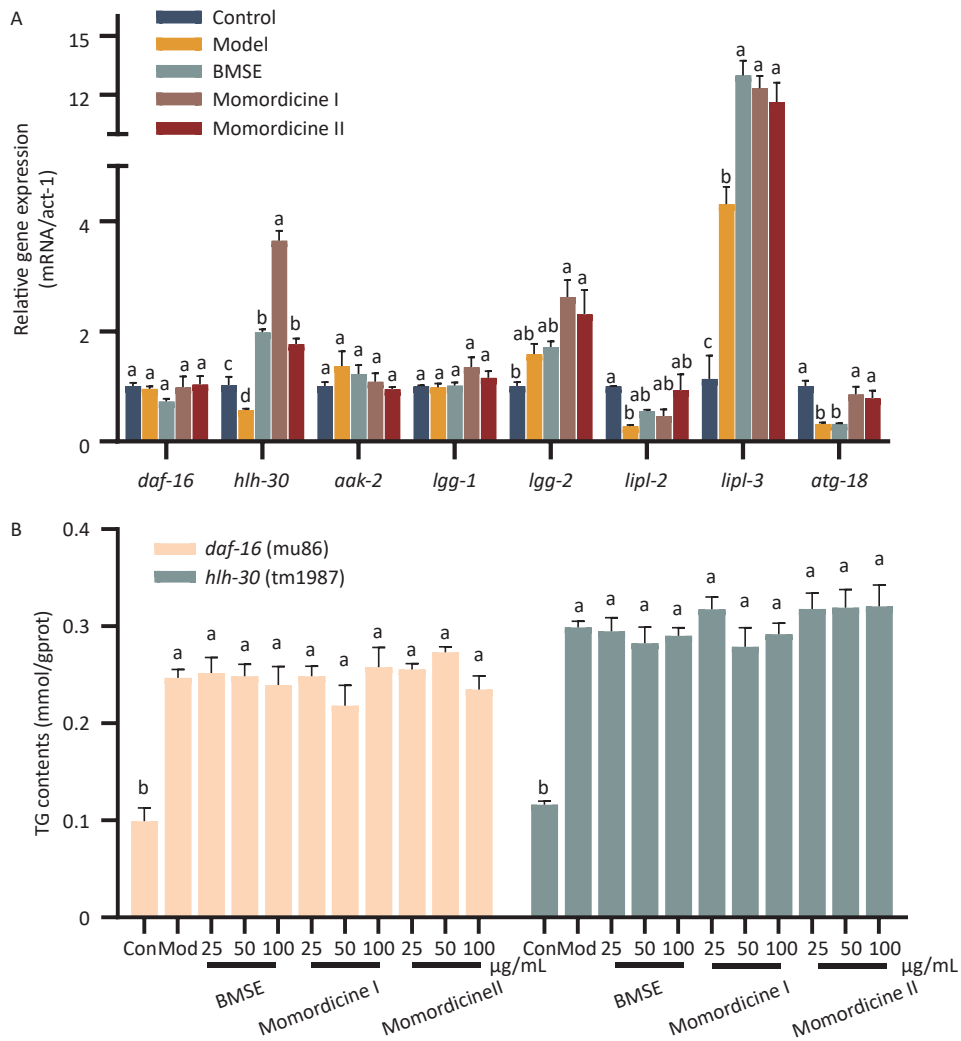

Figure 3. The molecular mechanisms of bitter melon saponin extract (BMSE) in alleviating fat accumulation. (A) N2 *Caenorhabditis elegans* (*C. elegans*) were pretreated with 100 μg/mL BMSE, Momordicine I and Momordicine II for 48 hours, and total RNA was extracted. The mRNA level was determined by qRT-PCR and normalized to the expression of *act-1*. (B) Triglyceride (TG) content in *daf-16* and *hlh-30 C. elegans* was measured. Results are showed as mean ± SEM. Values with different superscripts are significantly different (P < 0.05).

levels compared to the control group ($P < 0.05$), with BMSE, Momordicine I and Momordicine II treatments significantly counteracting this effect. Furthermore, *lipl-3* (LAL homolog), and *atg-18* (ATG-18 homolog) were markedly up-regulated in Momordicine I and Momordicine II groups, while other related genes including *daf-16* (FoxO1 homolog) showed no notable alterations. ATG-18 promotes the formation of autophagosomes, and LAL is the key enzyme hydrolyzing TG and cholesterol in lysosomes, both of which would be regulated by *daf-16* or *hlh-30*. No changes were registered in *daf-16* expression, however, our previous study indicating that *daf-16* and *hlh-30* possibly play a key role in lipid-lowering of BMSE leads us to speculate that *daf-16* is involved at the translation level rather than transcriptional level in the regulation of lipophagy by BMSE, though this requires further confirmation[2]. Whether Momordicine I and Momordicine II also act on *daf-16* and *hlh-30* in the same manner as BMSE still needs to be further verified with *C. elegans*.

The inhibition of fat accumulation in *daf-16* and *hlh-30 C. elegans* was diminished with the administration of BMSE, Momordicine I and Momordicine II, which combined with the previous study certifies that the saponins in bitter melon largely depended on *daf-16* and *hlh-30* to reduce fat storage[2] (Figure 3B). FoxO1 and TFEB could induce the expression of LAL, either alone or in combination, to promote autophagolysosome-mediated fat degradation and fatty acid β-oxidation[7]. Studies have revealed that FoxO1, as a significant regulator of the energy stress response, was directly regulated by the upstream target AMPK. TFEB was the major inducer of lysosomal biogenesis and autophagosome-lysosomal fusion, negatively regulated by the upstream target mTOR[8]. This re-confirmed that the AMPK/mTOR pathway speculated upon in network pharmacology played a key role in lipid reduction of BMSE, largely related to lipophagy. The diagram of the lipid-lowering mechanism of BMSE and its potential active compounds is shown in Supplementary Figure S4 (available in www.besjournal.com). In all, Momordicine I and Momordicine II alleviate fat deposition in obese *C. elegans via daf-16*/FoxO1 and *hlh-30*/TFEB mediated lipophagy.

In summary, based on LC/Q-TOF-MS/MS combined with network pharmacology, Momordicine I and Momordicine II were identified as the main compounds responsible for lowing lipid levels by BMSE. Experimental studies have verified that BMSE exerted remarkable lipid-lowering activity in obese *C. elegans*, along with Momordicine I, with 100 μg/mL providing the strongest effect. Most of all, Momordicine I and Momordicine II exerted their lipid-reduction capacity *via daf-16*/FoxO1 and *hlh-30*/TFEB mediated lipophagy, consistent with the KEGG predicted AMPK/mTOR signaling pathway. Overall, our findings not only identified pure compounds responsible for lipid-lowering effects in the ethyl alcohol extract of bitter melon, but also provided new insights into its underlying mechanisms. This study is expected to benefit the development of lipid-lowering products with clear efficacy and mechanisms.

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