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ASTAXANTHIN PREVENTS HEPATOSTEATOSIS AND HYPERLIPIDEMIA BUT NOT OBESITY IN HIGH-FAT DIET FED MICE

Duy Nguyen-Le[1,2,3,4+], Tien Ngo-My[12,3,4,+], Tuan Vo-Minh[1], Cao-Tri Nguyen[1], Thuoc Linh Tran[1,2,3,4], and Hieu Tran-Van[1,2,3,4,]*

Address(es): Dr. Hieu Tran-Van,
[1] Department of Molecular and Environmental Biotechnology, Faculty of Biology and Biotechnology, University of Science, Ho Chi Minh City, Vietnam.
[2] Laboratory of Biosensors, Faculty of Biology and Biotechnology, University of Science, Ho Chi Minh city, Vietnam.
[3] Laboratory of Molecular Biotechnology, University of Science, Ho Chi Minh city, Vietnam.
[4] Vietnam National University, Ho Chi Minh city, Vietnam.

*Corresponding author: tvhieu@hcmus.edu.vn

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ABSTRACT

Astaxanthin (AX) is a natural compound that regulates lipid metabolism in the liver, specifically in reducing hepatosteatosis. To the increment in Non-Alcoholic Fatty Liver Diseases (NAFLD) ratio and its burdens, prevention/treatment should address the world health problem. This study aimed to evaluate the ability of AX and its prolonged effects on the physiology of mice fed a high-fat diet. Mice fed with a high-fat diet (HFD) (n=12) were orally given AX at a dosage of 30 mg/kg body weight/day for 16 weeks, followed by eight weeks of AX termination, along with other CTL (normal chow diet) and HFD only group. At four time points of 8, 12, 16, and 24 weeks of the trial, three mice from each group were randomly dissected to collect blood, liver, and adipose tissue samples. AX given through oral gavage showed an excellent factor for maintaining total cholesterol, triglyceride, glucose, and Low-density Lipoprotein cholesterol (LDL-c). Moreover, AX did have impacts on preventing dyslipidemia in mice fed with HFD. Unexpectedly, AX supplied group caused excess weight gain in mice, shown in higher average body weight than HFD fed group. However, all the AX effects wore off after 8 weeks of termination. AX was a good player for liver and plasma lipid homeostasis, but not for weight control. Taken together, AX was a potential compound for preventing hepatosteatosis and hyperlipidemia, but not for controlling weight in mice fed with a HFD.

Keywords: Astaxanthin, dyslipidemia, diet-induced obesity, fatty liver, hepatosteatosis, hyperlipidemia, obese

INTRODUCTION

The liver is the largest and heaviest organ in the body. It is considered steatosis when the liver fat mass exceeds 5-10% of liver weight (**Wei *et al.*, 2008**). Fatty liver diseases have two types, including alcoholic and non-alcoholic fatty liver diseases. Alcoholic Fatty Liver Diseases (AFLD) occurs when the liver has to tolerate large amounts of alcoholic beverages absorbed into the body regularly. This type of disease often happens with alcohol dependence, which overloads the liver recovery function and leads to hepatic failure (**Arteel, 2003; Kato *et al.*, 2003**). The patient suffered from Non-Alcoholic Fatty Liver Diseases (NAFLD) when hepatosteatosis is caused by other aetiological agents such as obesity, type 2 diabetes, dyslipidemia, etc. (**Kneeman, Misdraji, and Corey, 2012**).
Overweight and obesity were the stages of body weight, determined by the Body Mass Index (BMI), where overweight people have a BMI of over 25 kg/m^2 and the obeses of over 30 kg/m^2 (according to WHO). Obese individuals reported to have drastic change in the hormone levels such as The hypothalamic-pituitary-adrenal (HPA) and hypothalamicpituitary-gonadal (HPG), which reduce the gonadotropin secretion and increase leptin. As a result, obese patient tend to have increase in abdominal and visceral fat deposits than overweight and normal people **(Ghaderpour *et al.*, 2022; Kim & Shin, 2020)**. In addition, studies demonstrated that obese individuals have 3.5-fold increased risk of developing NAFLD (**Li *et al.*, 2016**). The booming of the world obesity rate has brought along its related diseases, among which was the increment of NAFLD. In particular, an obese patient has 80 to 90 percent of progressing fatty liver diseases, especially NALFD (**Le *et al.*, 2017**). Indeed, obesity could be considered the major cause of NALFD, inflammation, and metabolic diseases (**Lu *et al.*, 2018**). Thus, it is essential to control the obesity rate rather than just handling it with NAFLD alone.
NAFLD includes Non-Alcoholic Fatty Liver (NAFL) and Non-Alcoholic Cirrhosis (Non-Alcoholic Steatohepatitis/NASH). NAFL usually does not progress to NASH (**Chalasani *et al.*, 2018**). Progression to NASH occurs only when a combination of fatty liver (NAFL) and inflammation (**Bhatt and Smith, 2015**). The degree of NALFDs ranged from simple steatosis to NASH with or without fibrosis. Steatotic hepatocytes were featured for balloon-like lipid droplets, called microvesicular or macrovesicular, in the liver cell cytoplasm (**Tiniakos, 2009**). The proportion of micro or macrovesicular in the liver declared the stage of steatohepatitis, which ranges from 5-33% for grade I, 33-66% for grade II and >66% for grade III (**Apica and Lee, 2014**). For instance, when the macrovesicular grow to their maximum size, they trigger necrosis process, thus leading to inflammation (**Brunt *et al.*, 1999**). As necrosis takes place, the release of matter inside adipocytes occurs, and macrophages are attracted to those decaying adipocytes. The presence of macrophages not only takes responsibility for phagocytosis, which focuses on cleaning up the cell remnants, but also for cytokine activation, the cause of inflammation (**Sun *et al.*, 2012**). This makes obesity, a physiological state in which the body accumulates exceeded fat, significantly related to NAFLD. In addition, other factors can cause inflammation, such as viruses, autoimmune, drugs, etc. The penetration of hepatitis A, B, C, D, and E viruses can stimulate the immune system causing inflammation in the liver (**Rossum *et al.*, 1998**). Drugs such as paracetamol or oral contraceptives, whose impacts are on hormone modulation, may directly or indirectly damage liver function and cause inflammation (**Laukkanen *et al.*, 2001**). The exact cause of NAFLD is still obscured, yet there are relations with the disease, as mentioned above. Mostly, the number of diseases related to NAFLD is diabetes and obesity as their linked mechanism.
Astaxanthin (AX) is a carotenoid with a dark red color and is found in microorganisms and some marine animals (salmon, shrimp, fish eggs, etc.). Still, the highest concentration of AX is found in green algae called *Haematococcus pluvialis*. Among carotenoids, AX is considered the best antioxidant and one of the most important elements in treating NAFLD. According to Jia's study, AX was good in reducing inflammatory factors, TNF-α and IL-6, both in plasma and liver (**Jia *et al.*, 2016**). Therefore, we could infer that AX was a potential substance for obesity control and NALFD prevention. However, in this concept, we would like to have further insights into how long these effects last. Animal models were proven to be the best fit for studying the bioactivities of compounds such as antifungal, antioxidant, or anti-inflammatory. In particular, induced mice models have recently been established to evaluate these bioactivities before being carried out in clinical trials (**Dehimat *et al.*, 2020; Ouda *et al.*, 2021; Shraideh *et al.*, 2020**). Therefore, based on the groundwork studies on induced mice models, we applied an obesity-induced mice model with an extremely high-fat diet (60% energy from fat) to assess the hepatosteatosis-preventing ability of AX.
In this study, we assessed the ability to reduce liver fat and blood lipids of AX *in vivo*, as well as the ability to suppress inflammatory factors. In the experiments, we combined a high-fat diet with oral administrated AX on Swiss mice (*Mus musculus*) and evaluated the ability to prevent NAFLD in the mouse model.

MATERIAL AND METHODS

Compliance with ethical standards

All procedures performed in animal studies followed the ethical standards of the institution in which the studies were conducted and with the approved legal acts of the Animal Care and Use Committee of the University of Science, VNU-HCM (AN: 09/18-0599-03).

Housing trial

Female Swiss mice (*Mus musculus*) (18-20 g, supplied by Institute of Drug Quality Control, Ho Chi Minh City, Vietnam) were randomly divided into three groups (n=12) including normal diet (CTL, supplied D12450J, Research Diets Inc., USA), high-fat diet (HFD, supplied D12492, Research Diets Inc., USA) and high-fat diet with AX supplementation (AX, 30 mg/kg body weight/day of astaxanthin, *Haematococcus pluvialis* Extract Powder, KAN Phytochemicals Pvt. Ltd., USA). Mice were maintained on a 12-hour light/dark cycle. The trial was divided into two phases. In the first phase, which prolonged 16 weeks, mice were orally administrated with water (CTL and HFD group) or AX (AX group); the second phase was prolonged for 8 weeks, at which AX supplementation was terminated, but still maintaining the same diet. Mouse body weight was measured, and food consumption was also determined daily. Dissection took place at weeks 8th, 12th, 16th, and 24th (n=3, at each time point) to collect blood samples, fat tissue, and liver. Before samples were collected, mice were fasted for 12 hours and were anesthetically injected with the composition of ketamine, xylazine, and PBS 1X with the volume ratio 1:0.5:3.5 (0.05 mL per each 10 g of body weight) to minimize their pain. Mice with either abnormal behaviors or conditions were separated, from which data were no longer recorded.

Evaluation of plasma lipid

Blood samples were centrifuged at 13,000 x g for 10 minutes to extract the serum for other tests. The semi-automatic biochemical Chem 5V3 machine then analyzed the obtained serum to measure cholesterol, triglyceride, high-density lipoprotein (HDL-c), Aspartate amino Transferase (AST) and Alanine amino Transferase (ALT), and glucose. Low-density lipoprotein (LDL-c) was calculated based on the following formula based on the measurement of High-density Lipoprotein (HDL-c), totalcholesterol, and triglyceride:

$$\mathrm{LDL_c} = \text{Total cholesterol} - \mathrm{HDL_c} - \frac{Triglyceride}{5}$$

Histological evaluation of liver

Mouse livers were cut into thin slices and fixed in 10% neutral formalin buffer for 24 hours. Slices were then embedded with paraffin to preserve. Parafinized samples were sliced into 10-µm-thin-slice and pasted on lime for the following staining procedure. The limes were washed three times with toluene, 5 minutes each time. Then the limes were immersed in 100º - 95º - 80º - 70º of EtOH, respectively. Samples were dipped in water 15 times, then stained with hematoxylin for 5 minutes. Before staining with 1% eosin, limes were washed with tap water, continued by dipping limes through 100º- 95º EtOH and toluene, respectively.

Liver slices were then observed under a microscope and images (more than three different sites for each slice) to calculate the proportion of droplets. Images were executed via ImageJ v1.4.3.x with "Analyzed Particle" option in "Measure" menu after going through "Brightness/Contrast" and "Threshold" options. The ratio of lipid droplets was measured by the fraction of (droplets area)/(total slice area).

Statistical analysis

All data are shown as the means ± SEM with three-time replications, and one-way Analysis of Variance (ANOVA) was used to analyze the significant differences between the groups. The graphs were made by Graphpad, and a p-value of less than 0.05 was considered significant. Additionally, ImageJ was used to analyze the presence of liver lipids in H&E stained liver sections.

RESULTS AND DISCUSSION

Evaluation of AX effect on mouse liver enzymes and histology

Non-Alcoholic Fatty Liver Diseases (NAFLDs) have become the main cause of liver cirrhosis and chronic liver diseases worldwide. Along with the rising burden of obesity, the ratio of chronic liver diseases is expected to increase (**Beste *et al.*, 2015; Mokdad *et al.*, 2014; Mokdad *et al.*, 2016**). With this situation, the mortality ratio of cirrhosis has been increasing (**Sepanlou *et al.*, 2020**). To overcome the issue, interfering with the progress of NAFLDs to liver cirrhosis was considered. Astaxanthin (AX) was a natural product reported to have effects on preventing the progression (**Ambati *et al.*, 2014**). In this study, we evaluated the effect of AX administration with 30 mg/kg/bodyweight dosage for 16 weeks and its post-termination for 8 weeks.

When the liver is steatosis, high concentrations of AST and ALT are secreted into the circulatory system. Therefore, the concentrations of AST and ALT enzymes were evaluated through a blood test to determine the presence of liver inflammation or hepatosteatosis.

Until the 12th week of the trial, both liver enzymes had not shown any difference between groups (P=0.57 for AST, P=0.18 for ALT) (Figure 1). A significant difference showed only in AST concentration at week 16th subsequently (P<0.01), whereas ALT did not show any significant difference except for AX at week 24th. Particularly, after 16 weeks, CTL and AX had considerably lower concentrations of AST compared to HFD (146.3 ± 34.0; 140.3 ± 22.0, respectively, compared to 302.2 ± 68.0 U/L of HFD) (Figure 1A). However, at the end of the trial, AST concentration was significantly higher than the level shown in the 16th week in CTL and AX but showed no difference among the three groups. As the changes happened, the AX supplement showed the ability to maintain normal liver function compared to HFD group. Specifically, the level of blood AST of CTL and AX increased after eight weeks of AX termination (from 146.3 ± 34.0; 140.3 ± 22.0 to 259.1 ± 38.0; 284.2 ± 21.0 U/L, respectively), indicating that the AX function in reducing hepatosteatosis was vanished, leaving the liver with no protection.

As for ALT level, at weeks 16th and 24th, there were no differences in CTL and HFD, except for AX, which had a risen ALT level after eight weeks of AX termination. The result suggested that there were injuries in mouse livers in AX group after the termination point, which showed in the leakage of ALT into the bloodstream. ALT is located mostly in liver tissue and is released into the circular system upon liver damage/injuries (**Gwaltney-Brant, 2016**). In figure 1, the AST and ALT ratio of CTL and HFD in the 16th and 24th weeks were higher than 0.8, and there were clues that CTL and HFD suffered from diet-induced metabolic disorders, which will be mentioned in later sessions. However, the ALT concentration among the three groups had not varied in a specific tendency, and we could only conclude that AX prevented liver from hepatitis steatosis, proven through AST reduction phenomena. This hepatoprotective action of AX may be due to some carotenoids' capacity to clear out reactive oxygen species, thus decreasing oxidative stress in hepatocytes (**Sindhu *et al.*, 2010**). Furthermore, carotenoids derived from marine lives proven to be good scavengers of reactive oxygen species (ROS) (**Rodrigues *et al.*, 2012**), which could be a support for the liver functional mantainance throughout the AX administration. AX mostly composed of β-carotene, the main compound which was reported to have reactions with ROS and acted as basis for reducing ALT and AST level (**Nishino *et al.*, 2017**). Other studies have revealed the concepts between plasma AST and ALT levels. The phenomenon was that ALT tended to be at low concentration in hepatitis fibrosis/cirrhosis individuals, where AST:ALT ratio was more than 1.0 rather than stayed high or fluctuated (**Sheth *et al.*, 1998; Williams & Hoofnagle, 1988**).

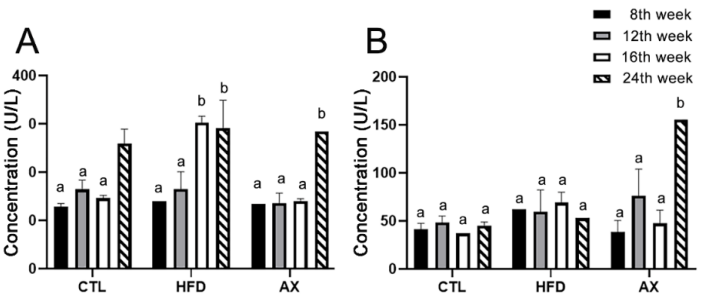

Figure 1 Plasma AST (A) and ALT (B) levels after 8, 12, 16, and 24 weeks. CTL, normal group; HFD, high-fat diet group; AX, high-fat diet with AX supplementation group. The data were presented as the means ± SEM, and significant differences were calculated using a one-way ANOVA test. Different letters (a and b) indicated significant differences between the three groups.

To demonstrate AX effects, images on H&E stained samples were taken and evaluated. The presence of balloon-like lipid droplets was only found in the case of HFD group at week 12th (Figure 2A). Until the end of the first phase, the AX group still showed no appearance of these droplets, while many of them could be seen in the other two groups. Although all samples were filled with droplets after the AX termination, AX group still maintained the lowest ratio among the three.

Then, we calculated the droplet ratio based on the area in each slice (at least three slices for each mouse), then the average ratio was determined. After 12 weeks of AX administration, the proportion of lipid droplet formation was different between groups (P<0.01) (Figure 2B). AX's was considered the lowest of all three groups, which was 0.04 ± 0.001%. Meanwhile, the proportion of CTL and HFD were 1.5 ± 0.6 and 2.3 ± 0.3%, respectively. In the 16th week of the trial, CTL and AX (1.90 ± 0.15; 0.20 ± 0.02%) showed no statistical difference compared to the 12th week (P=0.5 for CTL, P=0.57 for AX), which meant in 16 weeks of supplementation, AX prevented liver lipid accumulation.

In contrast, the percentage of HFD went dramatically from 2.3 ± 0.3% in week 12th to 21.3 ± 0.8% in week 16th. After eight weeks of AX termination, CTL and HFD (1.6 ± 0.2 and 20.70 ± 0.07%, respectively) appeared to have no significant change (P=0.51 for CTL, P=0.83 for HFD). Though having no significant lipid ratio, the

decline of HFD group could indicate those balloons were driven by necrosis. Meanwhile, the droplet ratio of AX rose significantly to 5.5 ± 1.0% (P<0.01), which supported that hepatosteatosis reducing the ability of AX no longer remained after termination. Taken together, AX is proven to maintain the liver from damage and lipid accumulation, as shown through the AST level and lipid droplets ratio.

The histologic results were in line with the enzymatic result, except CTL, which showed a high density of lipid droplets in the histopathology according to the level of AST after the 24th week. The difference might occur due to the difference in the food intake of CTL, which comprises a high amount of carbohydrates. The high carbohydrate diet was proven to raise the AST level higher than the high-fat, high-calorie diet, thus explaining the abnormal change in AST and ALT levels **(Agoun *et al.*, 2019; Purkins *et al.*, 2003**). Recent publications also reported the correlation of increased liver fat content with insulin resistance (**Hwang *et al.*, 2007**). Although we did not have measurements on the insulin levels among the groups, we supposed that this long-term carbohydrate diet raised the AST level of CTL in the 24th week and might also cause diabetes.

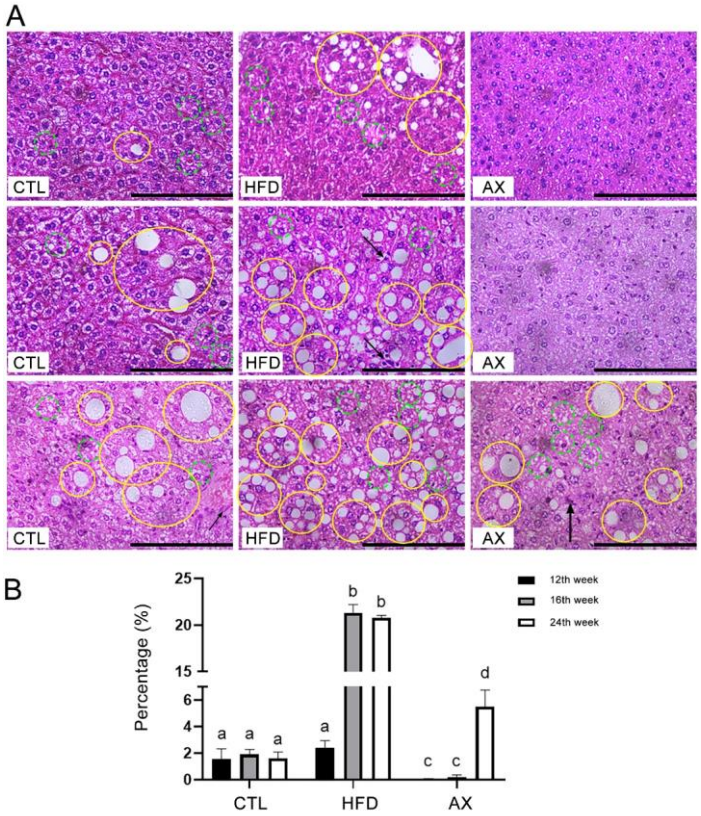

Figure 2 Histology liver stains at 40X magnification (A) and liver lipid droplets proportion (B) at 12 (top panels), 16 (middle panels), and 24 weeks (bottom panels) trial. CTL, normal group; HFD, high-fat diet group; AX, high-fat diet with AX supplementation group. The black lines in the lower right corner of each image represent 100 μm scale bars. Inflammatory foci (arrows): a cluster of more than 5 inflammatory cells; macrovesicular steatosis (yellow circles): large lipid droplets with a larger size than the nuclei presenting in hepatocytes; microvesicular steatosis (green dotted circle): small lipid droplets with a smaller size than the nuclei presenting in hepatocytes. The data were presented as the means ± SEM. Significant differences were calculated using a one-way ANOVA test. Different letters (a, b, c, and d) indicated significant differences between the three groups.

Evaluation of AX effect on plasma lipids and lipoproteins

To further assess the impact of AX on HFD-fed mice plasma lipids and lipoproteins, blood samples were investigated. In the first place, from the 8th to 12th week of the trial, cholesterol, triglyceride, HDL-c, and glucose level showed no statistical difference among groups (P=0.12 for cholesterol, P=0.8 for triglyceride, P=0.2 for HDL-c, P=0.5 for glucose). Only LDL-c had shown a significant difference since the 8th week (Figure 3 and Figure 4).

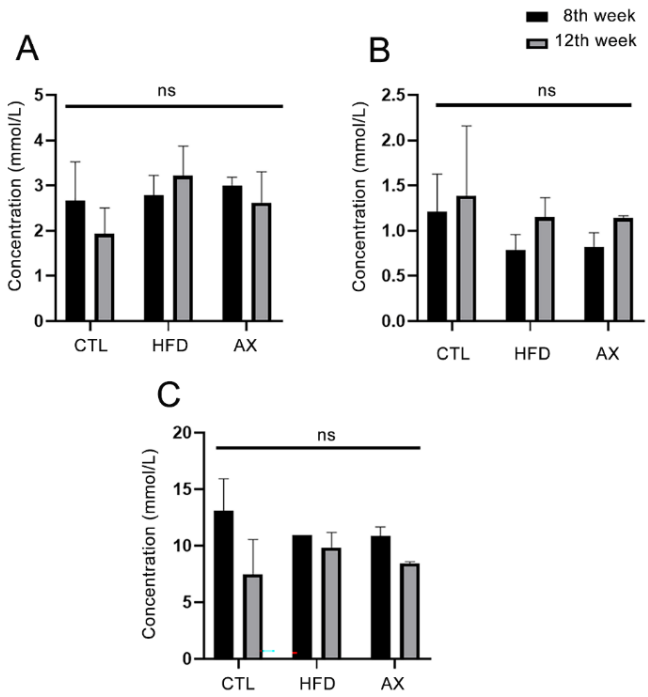

Figure 3 Cholesterol (A), triglyceride (B), and glucose (C) indexes of mouse groups after 8, 12 weeks trial. CTL, the normal group; HFD, high-fat diet group; AX, high-fat diet with AX supplementation group AST group. The data were presented as the means ± SEM. Significant differences were calculated using a one-way ANOVA test. *ns indicates no significant differences between the groups.

At weeks 16th and 24th, cholesterol and triglyceride of all three groups demonstrated significant changes between the two periods of time (week 16th, P<0.01 for cholesterol, P<0.01 for triglyceride, week 24th, P=0.03 for cholesterol, P<0.01 for triglyceride), only glucose among groups were not different after AX termination. Simultaneously, after eight weeks of termination, cholesterol, triglyceride, and LDL-c level of the groups were also statistically different between the 8-week-time (week 24th, P=0.03 for cholesterol, P<0.01 for triglyceride, P<0.01 for LDL-c) (Figure 4 and Figure 5).

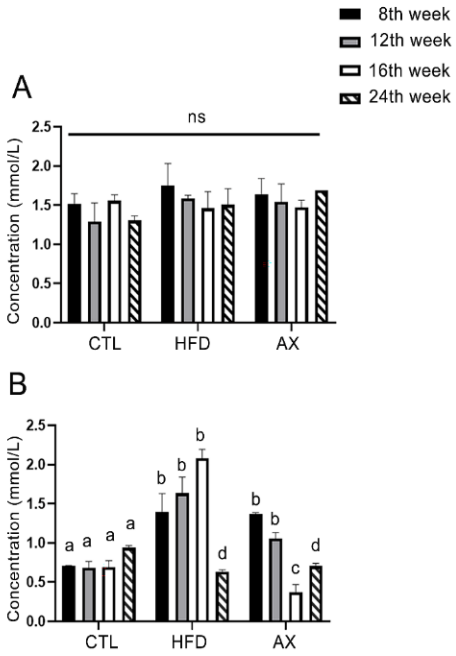

Figure 4 HDL-c (A) and LDL-c (B) indexes of mouse groups after 24 weeks of trial. CTL, the normal group; HFD, the high-fat diet group; AX, the high-fat diet with AX supplementation group. The data were presented as the means ± SEM. Significant differences were calculated using a one-way ANOVA test. *ns indicates no significant differences between the groups. Different letters indicate significant differences between the three groups.

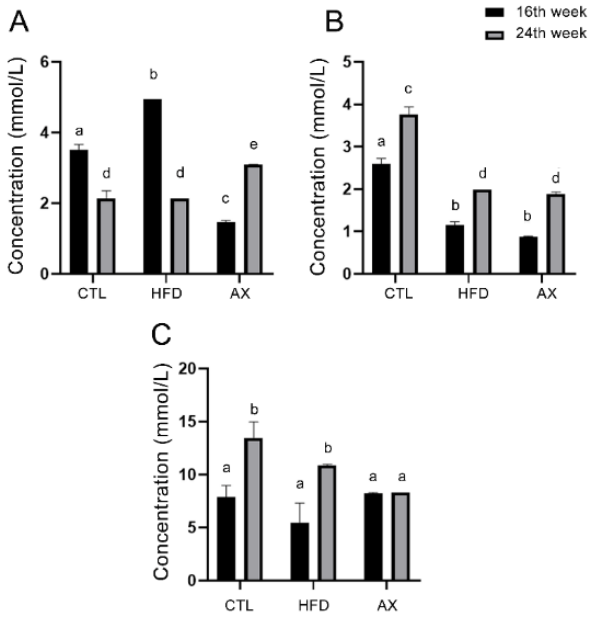

Figure 5 Cholesterol (A); triglyceride (B) and glucose (C) concentrations after 16, 24 weeks trial. CTL, normal group; HFD, high-fat diet group; AX, high-fat diet with AX supplementation group. The data were presented as means ± SEM, and significant differences were calculated using a one-way ANOVA test. Different letters indicate significant differences between the three groups.

As seen from figure 5, at week 16th, AX was the lowest in cholesterol and triglyceride (1.5 ± 0.01; 0.88 ± 0.01 mmol/L, respectively), proving the capacity of blood lipid control. After 24 weeks, both parameters had risen due to the loss of the AX effect. However, the cholesterol and triglyceride concentration of CTL and HFD had a noticeable change. The CTL and HFD cholesterol level decreased from 3.50 ± 0.02; 4.90 ± 0.01 in 16th week to 2.10 ± 0.04; 2.10 ± 0.01 mmol/L in 24th week and the triglyceride increased from 2.60 ± 0.01; 1.15 ± 0.01 in 16th to 3.77 ± 0.03; 1.99 ± 0.01 mmol/L in 24th. Besides that, CTL had the highest triglyceride level, 2.60 ± 0.01 mmol/L in the 16th week and 3.77 ± 0.03 mmol/L in the 24th week. As for these drastic changes, AX showed effects in maintaining blood cholesterol, as AX cholesterol levels were the lowest. In line with that, the AX glucose level was stable for the whole trial (Figure 4 and Figure 5), given that AX was a good factor for blood glucose preservation.

For lipoprotein concentration, LDL-c was different since the 8th week (Figure 4**)**. HFD's LDL-c was the highest from the 8th, 12th, and 16th weeks. Especially its peak reached 2.16 ± 0.01 mmol/L in the 16th week but declined after the termination phase. This fluctuation could relate to the total decrease in cholesterol (Figure 5) from the 16th to the 24th week due to diet-induced metabolic disorders, as mentioned before. Reversely, there was a gradual descent in LDL-c level of AX from the 8th to the 16th week, and the lowest value was 0.39 ± 0.02 mmol/L in the 16th week, although the opposite observation was shown after AX termination. Since LDL-c was considered to relate to cardiovascular diseases risks (**Miyajima *et al.*, 2018**), the result showed AX is also good for cardioprotection.

AX administration showed impacts on maintaining lipids and lipoproteins such as total cholesterol, glucose, and LDL-c level compared to HFD, demonstrating good outcomes for cardiovascular and NAFLDs. Likewise, AX termination tended to put aside almost of them. The plasma LDL-c lowering ability of carotenoids was reported in many studies, yet the exact mechanism has not been revealed. In line with the plasma LDL-c level, the presence of blood caroteinoid, particularly AX in this study, played a role in preventing the plasma cholesterol accumulation. Carotenoids, AX in particular, have been shown to protect LDL-c against oxidation, through scavenging free radicals (**di Mascio *et al.*, 1989**), and prevent the cholesterol accumulation (**Parthasarathy *et al.*, 1988; Quinn *et al.*, 1987**).

We theoretically deduced that the two groups (CTL and HFD) had suffered from diet-induced metabolic disorders, particularly diabetes because their glucose level had a rising tendency from week 16th to 24th. In 2018, there was a similarity to Perron's study about detecting the correlation between diet/energy balance and body weight through dietary in *Mus musculus* (**Perron *et al.*, 2018**). Blood glucose and insulin level are both elevated when taking a long-term HFD, which is the initial sign of diabetes type II that causes the decline of cholesterol and the ascendance of triglyceride (**Komeili *et al.*, 2016**). The highest triglyceride level of CTL group was due to the type of food consumed, and the increment of LDL-c was due to the loss of the AX effect after the 24th week. Importantly, the unchangeable HDL-c and descent LDL-c of AX within phase one of the trial showed reduced hyperlipidemia by lowering LDL-c level, which was a controversy with Jia's results as LDL-c was stable and HDL-c increased (**Jia *et al.*, 2016**). The discrepancy could theoretically be due to strain specific. Compared with the other two, AX showed better maintenance of plasma lipids and lipoproteins until the end of the AX administration phase.

Evaluation of AX effect on mouse weight and fat tissue

While the experiment progressed, we noticed an over-size state of AX-fed mice, especially from the 16th week. The mice administrated with AX appeared to be bigger in shape and size than CTL and HFD.

From weekly records, all three groups tended to gain weight equally in the first eight-week-time. From the 9th week, the weight of HFD and AX still escalated (35.0 ± 2.5 and 39.0 ± 3.0 g, respectively), especially with AX (39.0 ± 3.0 g) commenced to be different from the others, while the weight of CTL group seemed to be stable (32.0 ± 5.0 g). By the 12th week, AX's body weight (40.0 ± 2.0 g) began to differ from the other two and sustained until the 16th week of the experiment. After the first period, CTL and HFD were similar (34.0 ± 3.0 and 37.0 ± 1.0 g, respectively), except for the AX group, whose weight increased subsequently (Figure 6A). Overall, mice administered with AX gained weight and became obese faster than HFD, as the weight gain ratio of AX was the highest (Figure 6B).

Although proven to be a potential substrate for plasma lipids controlling, liver inflammation, and even lipid accumulation, AX seemed not fit as a treatment for NAFLD in obese patients because of the weight gain promotion. Studies by **Ikeuchi *et al.* (2006, 2007)** showed the benefits of weight controlling in AX treatment groups, while others found no differences in body weight with or without AX administration **(Jia *et al.*, 2016; Ni, 2015; Xie *et al.*, 2020**). The impact of diet-induced obesity/diabetes happened to be dependent on the mouse strain, thus could result in no differences between the high-fat or control group **(Montgomery *et al.*, 2013**). Hence, body weight/weight gain should not be considered an important factor of AX in diet-induced mice models. Moreover, in this study, the same high-fat diet was used compared with the study of **Ni *et al.* (2015)**, and we both came up with the result of non-weight-controlling in the AX treated group. Xie's study manifested the growth rate improvement of AX (**Xie *et al.*, 2020**). Therefore, whether AX can control weight gain or not could be concluded.

The result indicated that AX was a promising compound against NAFLD. However, obesity and related diseases still threaten global health, thus needing consideration. AX could effectively prevent NAFLD but promoted weight gain in mice fed a long-term high-fat diet, which suggests more studies for a novel natural product combined with AX to maintain relative weight gain in diet-induced obesity models.

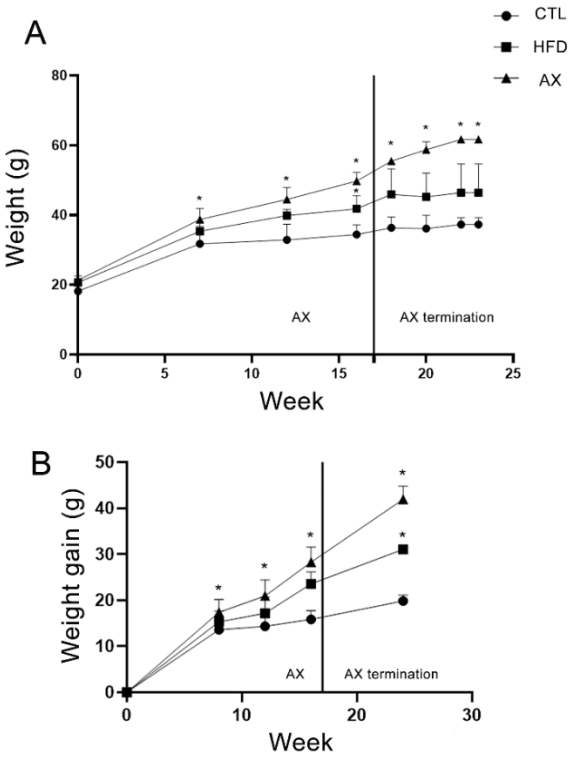

Figure 6 The weight progression (A) and the rate of weight gaining (B) of each mouse group through 24 weeks. CTL, normal group; HFD, high-fat diet group; AX, high-fat diet with AX supplementation group. * denoted statically different between groups.

CONCLUSION

AX was not only an excellent compound to prevent liver lipid accumulation and high blood cholesterol, but it was also capable of maintaining glucose levels, which was good against diet-induced metabolic diseases. Although AX promoted weight gain in the Swiss mouse model, it had shown effectiveness in biochemistry modulation. Furthermore, AX effects prompted to decline in the prolonged stage of AX withdrawal, which needed to be addressed for later applications. Altogether, more works need to be conducted in diabetes evaluation, such as insulin test and food choice for CTL, and weight controlling of AX, by combining with another natural compound to suppress weight gain.

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