Study on the hypolipidemic effect of *Moringa oleifera* seeds based on zebrafish hyperlipidemia model

Authors' contributions

This work was carried out in collaboration among all authors.

All authors read and approved the final manuscript.

Abstract:

Objective: Investigated the hypolipidemic effect of alcoholic extract of *Moringa oleifera* seeds using the zebrafish hyperlipidemia model. **Methods:** Using 0.3% egg yolk solution to feed zebrafish larval to construct the hyperlipidemia model. These larval fish in the model were treated with an extract of *Moringa oleifera* seeds for 2 days. Then, the tail vessels were observed after oil red O staining, and the accumulated optical density of lipids was analyzed. The body length, triglyceride (TG), and cholesterol (TC) levels were measured. Result: The accumulated optical density of lipids in the tail vessels of larval fish fed with 0.3% egg yolk was significantly higher than that of the blank group. The contents of TG and TC were also increased, indicating that the hyperlipidemia model was successfully established. After treatment with different concentrations of *Moringa oleifera* seed extract (10 μg/ml, 15 μg/ml, and 20 μg/ml), the staining attachment surface of vascular oil red O in the tail of hyperlipidemic zebrafish was significantly reduced by 42.8%, 63.3%, and 80.6% respectively. TG was reduced by 9.89%, 20.41%, and 26.40%. TC was reduced by 9.85%, 23.93%, and 33.80%. The data of each group were statistically significant ($P<0.01$). **Conclusion:** Moringa seeds had a significant hypolipidemic effect on zebrafish and had no significant effect on the skeletal growth length of zebrafish.

Keywords: zebrafish*;* hyperlipidemia; *Moringa oleifera* seeds; hypolipidemia.

1 INTRODUCTION

Moringa oleifera is also known as Chinese toon and drumstick, belongs to the family Moringaceae, genus Moringa Adans.*Moringa oleifera* seeds, originated in the Himalayan region of northern India, are the most commercially valuable varieties of the genus Moringa, which can be eaten, used and studied most[1]. It is a drought-tolerant and adaptable fast-growing tree species[2-3] named *Moringa oleifera* because of its pungent roots[4]. The roots, stems, leaves, flowers and seeds of *Moringa oleifera* are all high-quality health food materials[5-7].The seeds of the *Moringa oleifera* tree are rich in many active ingredients, such as oils, proteins, vitamins, and flavonoids[8-9]. Recent studies have pointed out its effectiveness in hypoglycemia[10], hypolipidemia[11], and hepatoprotection[12]. In this study, we investigated the efficacy of *Moringa oleifera* seeds in lowering blood lipids through a zebrafish hyperlipidemia model. This research can explore more effective options for applying this plant product in related nutraceuticals and foods.

2 MATERIALS AND METHODS

2.1 Materials and reagents

The zebrafish (AB strain, six months old) and brine shrimp were purchased from Shanghai Fei Xi Biotechnology Company. Oil Red O and 1-Phenyl-2-Thiourea(PTU) were purchased from Shanghai Aladdin Biochemical Technology Company. Propylene glycol, methylene blue, paraformaldehyde, and anhydrous ethanol were purchased from Chengdu Kelong Chemical Reagent Factory.

Atorvastatin calcium was purchased from Qilu Pharmaceutical Co. The total cholesterol and triglyceride test kit were purchased from Nanjing Jiancheng Biological Limited Engineering Institute.

2.2 Method

2.2.1 Hyperlipidemia model establish

The zebrafish were fed using the brine shrimp 3 times a day. In order to breed and hatch the larval, put male and female fish into a special breeding tank in the afternoon or evening, separate them with a clapboard, remove the baffle the following day, collect fish eggs after fertilization, and wash them. Next, pour larval culture solution (including methyl blue and PTU) into the culture dish and put it into the incubator at 28℃. During fish hatching, the culture solution should be changed every morning and evening, and hatching should be completed in 2-3 days. Several zebrafish larvae around 5 days post fertilization (dpf) were randomly selected and divided into three groups of equal numbers. The blank group was not fed, the control group was fed with brine shrimp, and the model group was fed with 0.3% egg yolk aqueous solution for 2 days. Furthermore, the enzyme activity and optical density were measured to judge whether the hyperlipidemia model was established successfully.

2.2.2 The Extraction of *Moringa oleifera* Seed

10g of hulled and properly crushed *Moringa oleifera* seeds. 200 ml of 70% ethanol was added into the powder and extracted at 90□ by reflux 3 times. The supernatant was filtered and concentrated by rotary distillation. The extract was dissolved with dimethyl sulfoxide(DMSO) to make 200 mg/ml of sample solution. Store in the refrigerator at 4□ for later use.

2.2.3 DMSO toxicity test

DMSO solutions of 0.05 μL/ml, 0.1 μL/ml, 0.25 μL/ml, 0.5 μL/ml, 1.5 μL/ml, and 4 μL/ml were prepared. 30 zebrafish of 6 pdf were placed at each concentration, treated for two days, and observed for mortality.

2.2.4 Determination of maximum tolerated concentration (MTC) of *Moringa oleifera* seed extract

180 zebrafish larval of 6dpf were randomly equally distributed in 6-well plates. The larval fish were fed with 10 ml of 100 μg/mL, 80 μg/mL, 50 μg/mL, 20 μg/mL, and 10 μg/mg of sample solution for 48 h. The toxicity phenotype and mortality of each group were observed.

2.2.5 Investigation of the hypolipidemic effect of *Moringa oleifera* seeds

After successfully establishing the model, zebrafish larval were equally distributed in five Petri dishes and given 0.24 μg/ml atorvastatin calcium (Positive control group), sample solution(10 μg/ml,15 μg/ml , 20 μg/ml *Moringa oleifera* seed extract, Sample group), and water (Blank group). The concentration of *Moringa oleifera* seed extract was lower than MTC. All groups were treated for 48h[13].

2.2.5.1 Lipid optical densitometry

The zebrafish larval were fixed overnight with 4% paraformaldehyde. The fish was washed using 1% PBS three times for 5 min to remove the fixative. After that, the fish were incubated with 3% Oleoresin O for 12h at room temperature and protected from light, and then washed and removed the excess Oleoresin O dye[14]. After decolorization, 10 zebrafish larval from each group were randomly selected and photographed under a light microscope, and data were collected. Image analysis was performed using image pro plus software(version 6.0)[15] to analyze and count the degree of oil red O staining in the tail vein of zebrafish. The above data will be used to evaluate the hypolipidemic effect of *Moringa oleifera* seed extract.

2.2.5.2 Body length measurement

The zebrafish larval was photographed with 0.5cm hair as a reference under the same frame.

2.2.5.3 Determination of triglyceride (TG) and total cholesterol (TC) content

Forty zebrafish larval were randomly selected from each group, and saline was added according to weight (g): volume (μl) = 1:10[16], and the supernatant was centrifuged for 10 min at 2500 rpm at 4°C.

The TG and TC contents were determined by referring to the method written in the instructions of the TG and TC kits.

3 RESULTS AND DISCUSSION

3.1 DMSO toxicity and maximum tolerance of *Moringa oleifera* seed extract

Table 1 shows that no significant toxic manifestations were observed in zebrafish larval after the DMSO concentration was less than 0.5 μL/ml.

Table 1 Survival of zebrafish at different concentrations of DMSO

Group No.	Zebrafish larval number	DMSO concentration(μL/ml)	Number of survivors
1	30	0	30
2	30	4	0
3	30	1.5	6
4	30	0.5	30
5	30	0.25	30
6	30	0.1	30
7	30	0.05	30

As shown in Table 2, no toxicity was observed at the concentration of 10-20 μg/ml of *Moringa oleifera* seed extract, with 30 survivors and 0% mortality; at the concentration of 30 μg/ml, 21 survivors and 30% mortality, and the concentration above 50 μg/ml, all zebrafish died.

Table 2 Survival of zebrafish larval at different concentrations of *Moringa oleifera* seed extract

Group No.	Zebrafish larval number	Sample concentration /μg/ml	Number of survivors
1	30	0	30
2	30	800	0
3	30	300	0
4	30	100	0
5	30	50	4
6	30	40	2
7	30	30	21
8	30	25	27
9	30	20	30
10	30	10	30

3.2 Results of hyperlipidemia modeling

As shown in Table 3, 0.3% egg yolk solution feeding (Model group) was significantly better than the brine shrimp feeding (Control group), which meant the hyperlipidemia model was successfully established.

Table 3 Analysis results of lipid optical density and TG\TC content in zebrafish larval after treatment with egg yolk powder solution

Group	Processing	Total tail vessel lipid optical density	TG content (mmol/L)	TC content (mmol/L)
Blank group	No feeding	0	0.250	1.061
Control group	Brine shrimp feeding	727615	1.035	2.809
Model group	Feeding 0.3% egg yolk solution	1750050	3.455	7.193

3.3 Results of the investigation of the hypolipidemic effect of *Moringa oleifera*

By comparing the oil-red O staining of zebrafish larval (Figure 1), the blank group had a lot of oil-red O attachment from the posterior gills to the caudal end. The positive control group only saw a small area

behind the gills attached by oil-red O. The sample group had oil-red O attachment from the posterior gills to the posterior end of the pectoral fin. However, the oil red O attachment area decreased with increasing concentration of *Moringa oleifera* seed extract.

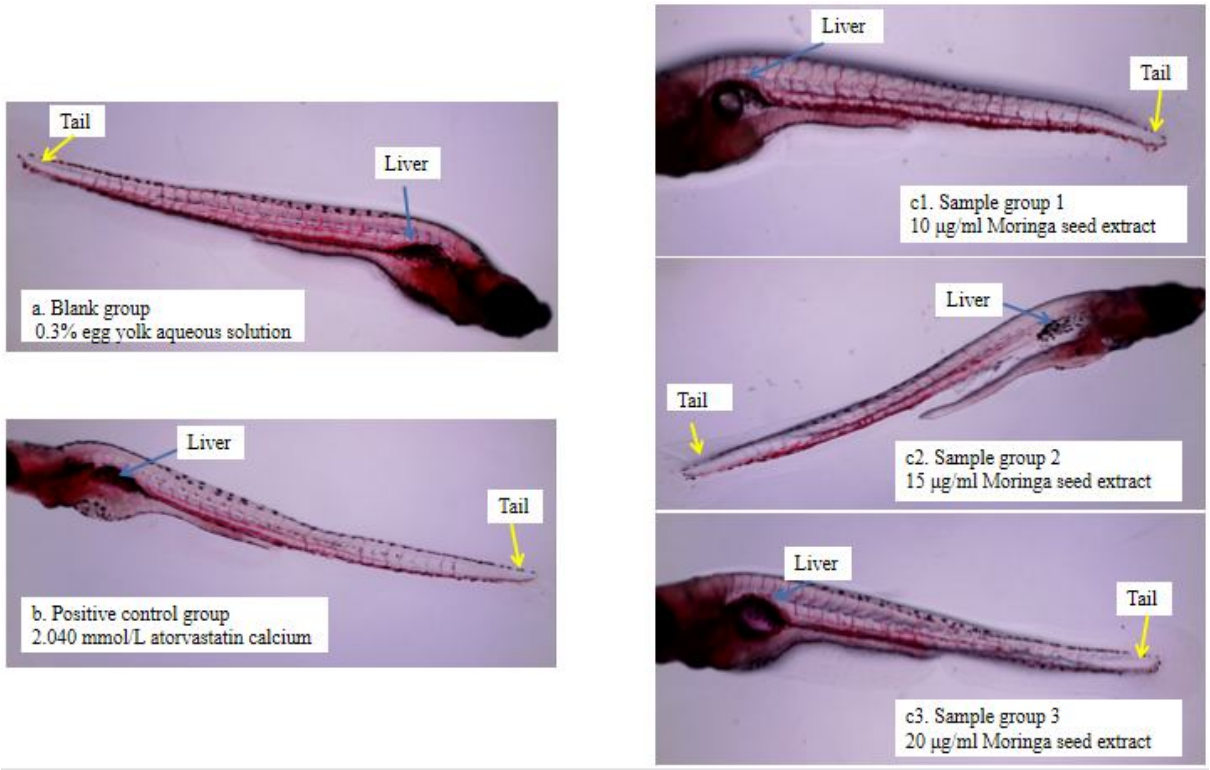

Figure 1 Comparison of oil red O staining of zebrafis larval

(The yellow arrows represent the caudal vessels attached by oil red O)

The cumulative optical density of each group was analyzed by Image Pro plus software, comparing the positive control group and the sample groups. The results (Table 5) indicated that atorvastatin calcium and moringa oleifera seeds have good lipid-lowering effects.

Table 4 indicated that zebrafish larval in 0.3% egg yolk aqueous solution could promote their skeletal growth compared to brime shrimp feeding. At the same time, no significant effect of M*oringa oleifera* seed extract was seen on their skeletal growth. The TG and TC levels were reduced in zebrafish larval after treatment with 2.040 mmol/L atorvastatin calcium and different concentrations of *Moringa oleifera* seed extract. It showed that M*oringa oleifera* seed extract could reduce zebrafish's triglyceride and cholesterol levels.

Table 4 Results of zebrafish larval's lipid, body length, TG and TC analysis after treatment in each group

Group	Processing	Blood lipid reduction rate (%)	Body length variation (%)	TG content reduction rate (%)	TC content reduction rate (%)
-	Brine shrimp feeding group	-	-	-	-
Blank group	0.3% egg yolk aqueous solution	-	8.83%	-	-
Positive control group	2.040 mmol/L atorvastatin calcium	89.90%	-	44.41%	23.93%
Sample groups	10 μg/ml Moringa seed extract	35.40%	0.15%	9.89%	9.85%

	15 μg/ml Moringa seed extract	58.50%	0.51%	20.41%	23.93%
	20 μg/ml Moringa seed extract	78.00%	0.20%	26.40%	33.80%

4 Conclusion

This study compared the red O staining, TG, and TC content of the hyperlipidemia zebrafish model treated with different concentrations of *Moringa oleifera* seed extract. The results showed significant differences in the degree of oil red O staining and the levels of TG and TC. The hypolipidemic effect of *Moringa oleifera* seeds on zebrafish was significant. The results provided a theoretical basis for developing future pharmaceuticals and health products centered on Moringa oleifera seeds in obesity.

ACKNOWLEDGEMENT

This research was supported by the Student Innovation Fund Program of Southwest University of Science and Technology (CX22-061, CX21-060) and Student Innovation Fund Program Targeted Poverty Alleviation of Southwest University of Science and Technology (JZ21-063).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Shuai HUA,Xiong DING,Haifeng CHEN,Jingjing SHI. Study on extraction technology of total flavonoids from Moringa oleifera seeds[J]. Chinese Journal of Ethnomedicine and Ethnopharmacy,2019,28(1):45-49.
2. Leone A,Spada, A,Battezzati A,Schiraldi A,Aristil J,Bertoli S. Cultivation, genetic, ethnopharmacology, phytochemistry and pharmacology of *Moringa oleifera* leaves: an overview[J]. International Journal of Molecular Sciences, 2015, 16(6): 12791-12835.
3. Nadeem M, Imran M. Promising features of *Moringa oleifera* oil: recent updates and perspectives[J]. Lipids in Health and Disease, 2016, 15(1): 212.
4. Ziji LIU,Jihua SUN ,Zhaohua LIU,Xuanmin DANG,Yan YANG . Application Value and Development Prospects Analysis of *Moringa oleifera* Lain[J]. Chinese Journal of Tropical Crops, 2014, 35(9): 1871-1878.
5. Ying YANG,Fan XIE,Shengxiang GONG,Wei ZHANG,Bei XU,Jinhong WU,Zhengwu WANG. Optimization of polyphenols extraction from Moringa oleifera Lam. seeds by response surface methodology and its antioxidant activity[J].Science and technology of food industry,2018,39(03):172-178.
6. Dongshun YANG, Jianlin FAN, Jinliang SHAO, Luxiang WANG. Comparative Analysis of Nutritional Components and Amino Acid Composition of Different Parts of Moringa oleifera Lam.[J].Journal of Shanxi Agricultural Science,2015,43(09):1110-1115.
7. Fengxia LIU,Miaomiao WANG,Youwei ZHAO,Xiaoyun XU,Siyi PAN. Extraction of Functional Components from Moringa oleifera and Development of Moringa oleifera-Based Products[J].Food Science,2015,36(19):282-286.
8. Xiaozhen HU,Ningping TAO,Changhua XU. Recent Advances in Research on the Medicinal and Culinary Value of *Moringa* Seeds [J].Food Science,2018,39(15):302-309.
9. Asghari G, Palizban A, Bakhshaei B. Quantitative analysis of the nutritional components in leaves and seeds of the Persian Moringa peregrina (Forssk.) Fiori[J]. Pharmacognosy Research, 2015,7(3): 242-248.
10. Tongtong DU. The chemical composition of Moringa seed and effective part of hypoglycemic[D].

Guangxi University of Chinese Medicine, 2018.
11. Xin CHEN, Yun WANG, Yongping ZHANG, Qin CHEN. Experiment study on the lipid lowering effect of Capsicum Leaf Lipid reducing granule on hyperesteremia mice[J]. Asia-Pacific Traditional Medicine, 2022, 18(2): 16-19.
12. Alaaeldin A. Hamza. Ameliorative effects of *Moringa oleifera* Lam seed extract on liver fibrosis in rats[J]. Food and Chemical Toxicology, 2010, 48(1): 345-355.
13. Xinyi LI,Heqing CHEN, Hua XIA,Sheng LIN,Hongcai SHANG,Guihua TIAN. Study on the Hypolipidemic Effects of the Fengjiaojiangzhifang for the Hyperlipidemia Zebrafish[J]. Modernization of Traditional Chinese Medicine and Materia Materia-World Science and Technology, 2020, 22(5): 1629-1635.
14. Kan CHEN, Changqian WANG, Yuqi FAN, Zhihua HAN,Yue WANG,lin GAO,Huasu ZENG. Application of oil Red O in the staining of lipids in Zebransh[J]. Chinese Journal of Histochemistry and Cytochemistr, 2016, 25(4): 358-360.
15. Guiyao WANG,,Wei LIU,,Meiping BAI,Jinping LI,Likuan LIU,WenMing ZUO,Yang ZENG. Investigating the efficacy of aqueous extract of green tea from golden dewberry as an aid to improve blood glucose and blood lipid levels based on zebrafish model[J]. Qinghai Science and Technology, 2022, 29(1): 91-96.
16. Fulin FAN, Yueqin CHEN, Mei ZHAO ,Lamei LIU. Effect and mechanism of ursolic acid in non-alcoholic fatty liver disease based on zebrafish model[J]. Natural Product Research and Development, 2020, 32(4): 645-651.