Original Article

Hypolipidemic effect of flavonoids from mulberry leaves in triton WR-1339 induced hyperlipidemic mice

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The aim of this study was to investigate the hypolipidemic effect of the total flavonoids from mulberry leaves (MTF) in hyperlipidemic mice. The total flavonoids have been isolated from mulberry (*Morus alba* L.) leaves and purified by macroporous resins. After that, the content of MTF is 581.7 mg g^{-1} in dried product. The hypolipidemic effect of MTF has been evaluated in triton WR-1339 (400 mg kg^{-1}) induced hyperlipidemic mice. The beneficial effects of MTF on serum lipid levels are more significant at 12h post MTF administration than at 6h. The levels of TG, TC and LDL-C were remarkably reduced to 388, 257 and 189 mg 100 ml^{-1} in MTF (30 mg kg^{-1}) and triton WR-1339 treated mice, compared with 540, 464 and 299 mg 100 ml^{-1}, respectively, in group treated by triton WR-1339 only. The ratios of HDL-C/TC and HDL-C/LDL-C were increased to 0.42 and 0.57 post MTF (30 mg kg^{-1}) administration, whereas these two ratios at the low levels of 0.33 and 0.52, respectively, in the reference group. These findings support a serum a lipid-favourable activity for mulberry leaf flavonoids.

Key Words: flavonoid, *Morus alba* L., hypolipidemic activity, triton WR-1339

Introduction

Hyperlipidemia is one of the greatest risk factors contributing to prevalence and severity of cardiovascular diseases. The epidemiologic data shows that the prevalence of dyslipidemia in Chinese adults aged 18 and above is 18.6%, which is to say the number of dyslipidemic patients has reached 160 million.[1] It is also reported that almost 12 million people die of cardiovascular diseases and cerebral apoplexy each year all over the world. Therefore, it is very important to pay attention to early stage prevention and control of hyperlipidemia in a comprehensive way. However, the risk of hyperlipidemia would be reduced by consumption of flavonoids and their glycosides, supported by abundant studies.[3-6] For instance, the flavonoids extracted from gingko, soybean, and some other plants have been reported as the antioxidants and could be beneficial to hyperlipidemia patients. *Morus alba* L. is a widely distributed plant in China, whose leaves, root bark and branches have long been used in Chinese medicine to treat fever, protect the liver, improve eyesight, facilitate discharge of urine and lower blood pressure.[20] Mulberry leaves have also been used in leaf stewing, which is considered as a healthy drink on weight reducing. Moreover, the aqueous extract of mulberry leaves, rich in flavonoids, acts as the scavenger of blood lipid radicals in our previous study on sugar metabolism and antioxidation in diabetic rats.[2] Hereby, the aim of this study was to separate the total flavonoids from mulberry leaves (MTF) and to detect whether the consumption of MTF could improve the serum lipid status in hyperlipidemic mice. In this study, The NKA-9 type of macroporous resins has been used for separation and purification of flavonoids, and the hypolipidemic effect of MTF has been measured in hyperlipidemic mice model induced by triton WR-1339.

Materials and methods

Extraction and purification of flavonoids from mulberry leaves

Dried leaves from *Morus alba* L. were used for extraction of flavonoids. Identification of plant was verified by associate professor Yuelan Li, Department of Pharmacy, Zhejiang College of Traditional Chinese Medicine. Powdered material was extracted twice with 70% ethanol solution (v/v) at 90 ℃ for 2h.. After filtration and centrifugation (3000 rpm, 15 min), the solvent was evaporated and the aqueous extract was condensed under reduced pressure. The NKA-9 macroporous resins were chosen to separate and purify the MTF from the crude extract.[7] The MTF solution was diluted to 1.5 mg ml^{-1} and stored at 4 ℃ before use.

Determination of flavonoids

The UV-2300 spectrophotometer (Teckcomp, China) was used to determine the content of flavonoids in the above isolated product at 510 nm.[8]

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The flavonoid content was calculated using the following linear equation based on the calibration curve that prepared by rutin, range from 2.0 μg ml^{-1} to 60 μg ml^{-1}.

A = 0.005 + 0.0116C, r = 0.9998

Where A is the absorbance
C is the flavonoid content in μg ml^{-1}.

Experimental animals

ICR mice, 5 weeks old, weighing 20 ± 2 g, were purchased from the Institute of Experimental Animals, Academy of Medical Sciences (Hangzhou, China) and were acclimatized for 1 week before experiment. The animals were housed in individual cages with free access to water and regular ad libitum, in a controlled environment maintaining a 12h light - 12h dark cycle, the temperature of 24 ± 1 ℃, and the humidity of 55 ± 10 %.

All the mice were randomly divided into 6 groups when experiments began: normal control group, model group, MTF treatment groups (7.5, 15 and 30 mg kg^{-1}) and positive control group.

Design

All mice except normal control group were injected with triton WR-1339 (Sigma, USA) at a dose of 400 mg kg^{-1} to achieve the hyperlipidemic animal models, while normal control group was injected with normal saline (NS) of the same volume.[9, 10] The triton WR-1339 was dissolved in NS to a final concentration of 4%. Twelve hours following the triton WR-1339 injecting, MTF groups were treated by MTF at doses of 7.5, 15 or 30 mg kg^{-1} respectively, orally by gastric intubation. Simultaneously, the positive control group was given ginkgetin at a dose of 15 mg kg^{-1} by intragastric administration.. The serum samples were separated from the mouse blood samples, collected from the retro-orbital plexus at 12, 18 and 24 h after triton WR-1339 injection, and stored at -18 ℃ before determination.

Determination of serum lipid levels

The amount of total cholesterol (TC), triacylglycerol (TG), high density lipoprotein cholesterol (HDL-C) and low density lipoprotein cholesterol (LDL-C) in mouse serum were assayed by enzymatic kits (Rongsheng Biotech, China); ratios of HDL-C/TC and HDL-C/LDL-C were calculated to describe serum lipid levels.[9]

Statistical analysis

All the data were expressed as mean ± S.D. Statistical significance was calculated using one- way analysis of variance (ANOVA). Significance was accepted at the $p<0.05$.

Result

Qualitative analysis of MTF

The flavonoids content was 28.7 mg g^{-1} in mulberry leaves, and 581.7 mg g^{-1} in dried product post purification by NKA-9 macroporous resins.

Table 1. Effect of MTF on serum lipid levels in mice 18h post triton WR-1339 injection

	Normal	Triton WR-1339	MTF treatment (mg kg^{-1})			Ginkgetin (15 mg kg^{-1})
			7.5	15	30	
TG (mg 100 ml^{-1})	85.7± 5.9	607± 50 #	596± 59	553± 54 *	438± 38**	542± 47**
TC (mg 100 ml^{-1})	79.1± 5.4	441± 30 #	413± 41	361± 35**	285± 27**	370± 32**
LDL-C (mg 100 ml^{-1})	72.1± 5.1	287± 24 #	273± 25	247± 26**	210± 16**	248± 20**
HDL-C/TC	0.52± 0.04	0.34± 0.03 #	0.34± 0.03	0.36± 0.03	0.39± 0.04**	0.36± 0.03
HDL-C/LDL-C	0.57± 0.05	0.52± 0.06 #	0.52± 0.04	0.53± 0.05	0.53± 0.06	0.54± 0.06

n =10; Mean ± S.D. # $p<0.01$ compared with control group. * $p<0.05$, ** $p<0.01$ compared with model group.

Table 2. Effect of MTF on serum lipid levels in mice 24h post triton WR-1339 injection

	Normal	Triton WR-1339	MTF treatment (mg kg^{-1})			Ginkgetin (15 mg kg^{-1})
			7.5	15	30	
TG (mg 100 ml^{-1})	84.5± 5.4	540± 40 #	526± 46	494± 46 *	388± 26**	452± 38**
TC (mg 100 ml^{-1})	81.1± 4.9	464± 37 #	421± 44 *	339± 33**	257± 25**	345± 40**
LDL-C (mg 100 ml^{-1})	72.7± 5.5	299± 26 #	276± 22 *	229± 17**	189± 16**	238± 19**
HDL-C/TC	0.52± 0.03	0.33± 0.03 #	0.34± 0.03	0.38± 0.04**	0.42± 0.04**	0.37± 0.04*
HDL-C/LDL-C	0.58± 0.05	0.52± 0.04 #	0.52± 0.07	0.56± 0.04	0.57± 0.06*	0.53± 0.06

n =10; Mean ± S.D. # $p<0.01$ compared with control group. * $p<0.05$, ** $p<0.01$ compared with model group.

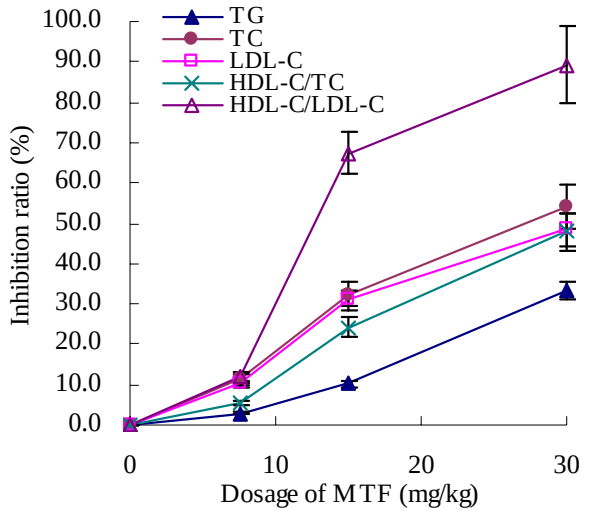

Figure 1. The inhabitation of MTF in serum lipid levels. The serum lipid levels were resisted by MTF, at the doses of 7.5, 15 and 30 mg kg^{-1}, 24h post triton WR-1339 injection. Date is expressed as mean± S.D. obtained from ten mice in each group.

Hypolipidemic effect of MTF

The levels of serum lipids were significantly increased approximate 7 folds in TG, 5 folds in TC and 4 folds in LDL-C in triton WR-1339 induced hyperlipidemic mice, whereas the ratios of HDL-C/TC and HLD-C/LDL-C were decreased by 40% and 10%, respectively, compared with the normal control. These changes can be inhibited or reduced by MTF, suggesting the hypolipidemic effect of MTF (Table 1 & Table 2).

As shown in Table 2, 24 hours following the triton WR-1339 injection (400 mg kg^{-1}), the amounts of TG, TC and LDL-C were remarkably reduced to 388, 257 and 189 mg 100 ml^{-1} in MTF (30 mg kg^{-1}) and triton WR-1339 treated mice, compared with 540.1, 464 and 299mg 100 ml^{-1}, respectively, in group treated by triton WR-1339 only. The ratio of HDL-C/TC was obviously raised to 0.38 and 0.42 in MTF treatment groups, at the doses of 15 and 30 mg kg^{-1}, while the ratio was depressed to 0.33 in mice treated with triton WR-1339 alone. Despite the high HLD-C/LDL-C ratio present in all the MTF treatment groups, there was no significant difference between

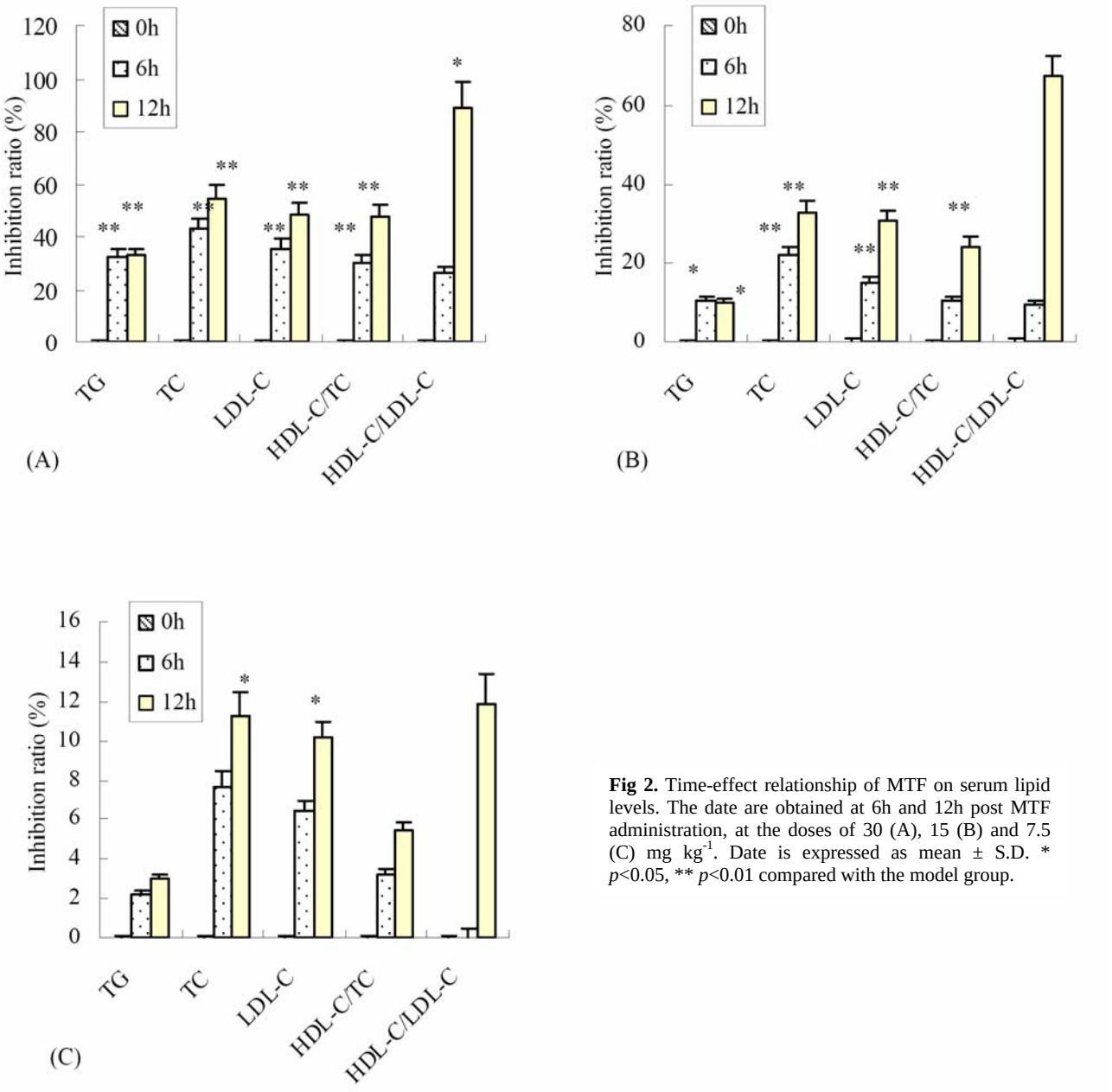

Fig 2. Time-effect relationship of MTF on serum lipid levels. The date are obtained at 6h and 12h post MTF administration, at the doses of 30 (A), 15 (B) and 7.5 (C) mg kg^{-1}. Date is expressed as mean ± S.D. * $p<0.05$, ** $p<0.01$ compared with the model group.

groups of either 7.5 mg kg^{-1} dose or 15 mg kg^{-1} dose and groups without MTF treatment.

Both Table 1 and Table 2 indicated a dose dependent hypolipidemic activity of MTF, which was observed more clearly in Fig.1. The increases in serum TG, TC, LDL-C induced by triton WR-1339 and declines in HDL-C/TC, HLD-C/LDL-C ratios were all resisted by MTF co-treatment. The inhibition ratios of TG, TC, LDL-C, ratio HDL-C/TC and HLD-C/LDL-C, as described in Fig.1, were dose dependent.

Time - effect relationship of MTF on serum lipid levels

when MTF (30 mg kg^{-1}) was given to the hyperlipidemic mice induced by triton WR-1339 (400 mg kg^{-1}), the hypolipidemic effect acted continually for 12 hours at least (Fig 2A). Similar phenomenon could be observed at the dose of 15 mg kg^{-1} (Fig 2B). But the difference is not significantly enough to leap to a conclusion at the dose of 7.5 mg kg^{-1} (Fag 2C). It can also be observed that the lipid lowering activity of MTF is more efficacious at 12h post MTF administration than at 6h.

Discussion

In Chinese traditional medicine, mulberry leaves have been used in protecting liver, improving eyesight, facilitating discharge of urine, lowering blood pressure and controlling weight. In our prior study, when we were interested in the effect of MTF on sugar metabolism and antioxidation in diabetic rats, we found that MTF was the scavenger of lipid free radicals,[2, 11] indicating a potential lipid lowering activity of MTF. To verify this feature of MTF, triton WR-1339, a nonionic surfactant, was used in this study to generate hyperlipidemic animal models.[10, 12, 13]It is well known that high serum TC, TG and LDL-C levels are primary risk factors for vascular diseases, and high serum level of HDL-C confers a protective effect against its development. The experimental data in the current study shows that all the changes in serum lipid levels induced by triton WR-1339 can be resisted by MTF. The amount of TG and TC could be obviously reduced by MTF in a dose dependent manner. Meanwhile, the proportion of cholesterol component could also be changed under the action of MTF. These results prove the hypolipidemic activity of MTF. MTF may become a kind of functional food or even natural drug in future.

The oxidative modification of LDL and its accumulation in serum is a primary event in the proceeding of atherosclerosis.[14] It is generally believed that flavonoids or vitamin antioxidants, which increase LDL oxidation resistance of the body, could inhibit atherosclerosis, though there is no direct evidence yet.[15] It was reported that some isoflavones increase LDL oxidation resistance, like soybean isoflavones and genistein derivatives.[15] The flavonoids extracted from Mulberry leaves, are identified as efficacious antioxidant compounds, taking quercetin, keampferol and their glycosides for instance. The strong LDL antioxidant activities of quercetin 3-(6- malonylglucoside), rutin (quercetin 3-rutinoside) and isoquercitrin (quercetin 3-glucoside) were demonstrated in vitro.[16]

The content of HDL-C in serum implies the activity of lecithin cholesterol acyltransferase (LCAT), which plays a key role in lipoprotein metabolism and may contribute to the regulation of blood lipids.[17] Allowing for abnormal changes of HDL-C in triton WR-1339 induced acute animal models, we chose HDL-C/TC and HDL-C/LDL-C ratios instead.[9] The two ratios represent the proportion of cholesterol component and may provide valid indices for identifying individuals at risk of peripheral arterial diseases.[18] Flavonoids may decrease the risk of cardiovascular disease by increasing these two ratios. Flavonoids increase the HDL-C/LDL-C ratio that may hasten removal of cholesterol from peripheral tissues to liver for catabolism and excretion.[19] In our study, the ratio HDL-C/TC was substantially boosted in MTF treated mice, at doses of 15 and 30 mg 100 kg^{-1}. Though there was no statistical significance, the ratio HDL-C/LDL-C was raised as well.

In conclusion, the results of the experiments above provide useful information regarding the lipid lowering activity of flavonoids extracted from mulberry (*Morus alba* L.) leaves and purified by macroporous resins. The beneficial effects of MTF on serum lipid levels are dose dependent and time dependent. The current study supports, at least partly, the traditional use of this ethnomedical plant. The mechanisms responsible for this hypolipidemic effect of MTF should be explored further in future studies.

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