August 2008 Volume 4 Issue 2

Trade Science Inc.

Natural Products

An Indian Journal

Full Paper

NPAIJ, 4(2), 2008 [132-139]

Anti-hyperlipidaemic activity of *Ziziphus jujuba* mill

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Received: 26th June, 2008 ; Accepted: 1st July, 2008

ABSTRACT

In the present study, methanolic extract (MEZJ) from *Ziziphus jujuba* mill leaf powder was evaluated for its hypocholesterolaemic and hypotriglyc-eridaemic activities using Triton WR-1339 induced hyperlipemic rats as experimental model. Hyperlipideamia was developed by intraperitonial injection of Triton (200 mg/kg body weight). The animals were divided into Group-I : Animals received 1% CMC, p.o Group - II: Animals received Atrovastatin 30 mg/kg, p.o Group -III : Animals received MEZJ at 125 mg/kg, p.o Group -IV : Animals received MEZJ at 250 mg/kg, p.o Group - V: Animals received MEZJ at 500 mg/kg, p.o All the animals were treated with their respective extracts / drug twice a day orally according to their groups for seven days. On the seventh day all the animals were deprived of food and water *at libidum* for 18 hours. Triton WR-1339 at the dose of 350 mg / kg was injected intraperitoneally to all the rats. After the triton injection blood was collected immediately from all the animals through retro orbital puncture technique at various time intervals like 0 hr, 6 hr and 24 hr respectively. Serum was separated by centrifuging the blood the supernatant was used for the following investigations. Total cholesterol, LDL, Glucose, Triglyceride, Glutathione Nitrite and Glutathione peroxidase (GPx) by standard procedures. The MEZJ treated animals at doses (125, 250, 500 mg) showed significant ($P<0.05$, $P<0.01$, $P<0.001$) protection against elevated cholesterol levels when compared to control group of animals. And also a better response over the other parameters mentioned above. Therefore the increased enzyme activities resulting from the treatment with MEJZ may prevent the oxidative damage by detoxification reactive oxygen species and inhibiting lipid peroxidation thus reducing hyperlipidemia. Further studies are required to isolate the active principle present in *Ziziphus jujuba* Mill and evaluate its pharmacological activity, which may give a potential hyperlipidemic drug.

INTRODUCTION

The plant *Ziziphus jujuba* mill is a small subdeciduous tree with dense spreading crown grows up to a height of 6 m. The color varies from black to grey or brown belonging to the family - Rhamnaceae. The plant has been used in various ailments like biliousness and headache (Root). Bark: to treat diarrhoea. Leaves: biliousness, diarrhoea, antipyretic, reduce obesity. It is used as anthelmintic. Fruit:The ripe fruit is cooling, indigestible, aphrodisiac and tonic. useful in fever and for wounds and ulcers. The unripe fruit removes "vata" and causes "kapha". It also increases thirst, lessens expectoration and biliosness. The dry fruit is laxative and appetiser, removes impurities form blood, allays thirst. The fruit is said to be mucilaginous, pectoral and styptic. According to folklore claim, the plant *Ziziphus jujuba* mill is used in the treatment of hypercholesterolemia. But there exists no evidence relating to the modern scientific methods of evaluation. Hence the present study is aimed to develop a drug from plant origin to treat hypercholesterolemia. This pilot study is aimed at proving the plants hypercholesterolemic activity using standard experimental animal model.

MATERIAL

Plant collection and authentication

The plant material of *Zizyphus jujuba* was collected in and around Chennai in the month of July 2007. The plant was identified and authenticated by Plant Anatomy Research Centre (PARC), Chennai. The voucher specimen of the plant was deposited at Plant Anatomy Research Centre-Chennai.

Extraction

The fresh leaf was dried under shade and coarsely powdered. The powder was passed through a 40-mesh sieve and was subjected to continuous maceration in methanol (90% v/v). The filtrate was distilled under reduced pressure using a rota flask evaporator until all the solvent had been removed to give the extract. The yield is 5.7% w/w. The extracts were used for preliminary phytochemical screening. The methanolic extract (MEZJ) was alone used for the pharmacological studies.

The methanolic extract was administered to the animals by dissolving each time with 1% CMC.

Experimental animals

Adult Wistar rats of either sex weighing 180-250 g were used in the pharmacological studies. The animals were maintained in day and night rhythm in a well-ventilated room. The temperature was maintained at 22 ± 1°C with humidity at 55 ± 5 %. They were fed balanced rodent pelleted diet from Poultry Research Station, Nandanam, Chennai-35, and tap water *ad libitum* throughout the experimental period. The animals were housed for one week, prior to the experiments to acclimatize to laboratory temperature. The experimental protocol was approved by the Institutional Animal Ethics Committee IAEC

Procedure

The rats were divided into five groups each group consisting of six animals and treated as follows. Group - I : Animals received 1% CMC, p.o Group - II: Animals received Atrovastatin 30 mg/kg, p.o Group -III : Animals received MEZJ at 125 mg/kg, p.o Group -IV : Animals received MEZJ at 250 mg/kg, p.o Group - V: Animals received MEZJ at 500 mg/kg, p.o All the animals were treated with their respective extracts / drug twice a day orally according to their groups for seven days. On the seventh day all the animals were deprived of food and water *at libidum* for 18 hours. Triton WR-1339 at the dose of 350 mg / kg was injected intraperitoneally to all the rats. After the triton injection blood was collected immediately from all the animals through retro orbital puncture technique at various time intervals like 0 hr, 6 hr and 24 hr respectively. Serum was separated by centrifuging the blood at 1500 rpm for 10 minutes and the supernatant was used for the following investigations.Total cholesterol was assayed in serum by using a standard total cholesterol kit method[1]. LDL was assayed in serum by using a standard LDL kit method[2]. Glucose was assayed in serum by using a standard glucose kit method [3]. Triglyceride was assayed in serum by using a standard triglyceride kit method[4].Glutathione content was estimated by 0.25 ml of serum was added to equal volume of ice cold 5% TCA. The precipitate was removed by centrifugation at 4000 rpm for 10 minutes. To 1 ml aliquot of supernatant, 0.25 ml of 0.2 M phosphate buffer, pH 8.0 and 0.5 ml of DTNB (0.6 mM in 0.2 M phosphate buffer, pH 8.0) was added and mixed well. The absorbance was read at 12 nm using spectrophotometer. The values were expressed in μg/dl serum[5]. Estimation of Nitrite was assayed by taking 0.2 ml of 10% homogenate followed by addition of 1.8 ml of saline and 0.4 ml of SSA for protein precipitation. The precipitate was removed by centrifugation at 4000 rpm for 10 minutes. To 1 ml aliquot of supernatant, 2 ml griess reagent was added and mixed well. The mixture was allowed to stand for 20 minutes under dark conditions. The colour intensity of the chromogen was read at 540 nm[6]. Superoxide dismutase was assayed by taking 0.05 ml of serum followed by addition of 0.3 ml of sodium pyrophosphate buffer (0.025 M, pH 8.3), 0.025 ml of PMS (186 μM) and 0.075 ml of NBT (300 μM in buffer of pH 8.3) The reaction was started by addition of 0.075 ml of NADH (780 μM in buffer of pH 8.3). After incubation at 30°C for 90 seconds, the reaction was stopped by addition of 0.25 ml glacial acetic acid. Then the reaction mixture was stirred vigorously and shaken with 2.0 ml of n-Butanol. The mixture was allowed to stand for 10 minutes and centrifuged. 1.5 ml of n-butanol alone was served as blank. The colour intensity of the chro-

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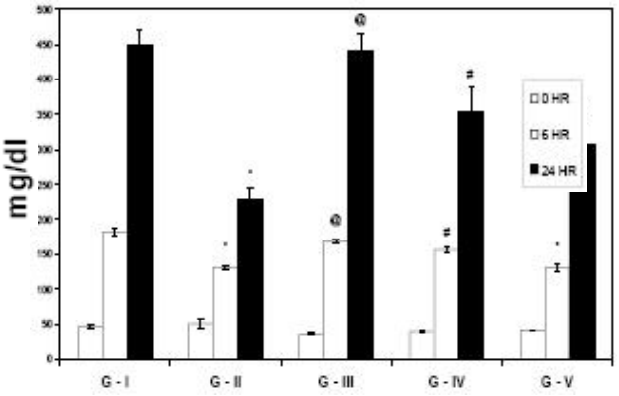
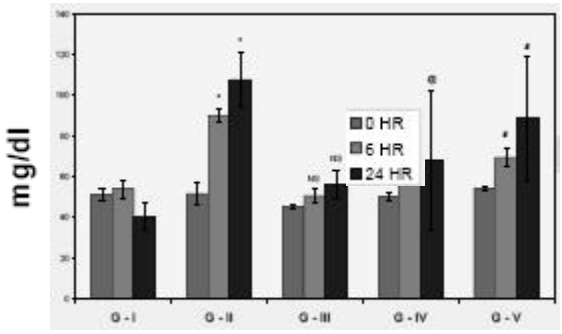


Group	Total Cholesterol (mg/dl)		
	0 hr	6 hr	24 hr
I	45.6 ± 2.5	182.1 ± 4.3	449.3 ± 22.6
II	50.2 ± 5. 7	131.1 ± 3.5*	228.2 ± 17.1*
III	35.6 ± 1.07	168.9 ± 3.6$^{@}$	440.4 ± 23.9$^{@}$
IV	39.6 ± 1.96	156.5 ± 3.6$^{\#}$	354.5 ± 33.6$^{\#}$
V	40.8 ± 1.21	130.9 ± 4.7*	308.2 ± 31.5*

Comparisons were made between: Group I vs II, III, IV, V, Values are expressed as mean ± SEM of 6 animals, Symbols represent statistical significance: * - P < 0.001, # - P < 0.01, @ - P < 0.05

Figure 1 : Total cholesterol level in serum



Group	HDL- Cholesterol (mg/dl)		
	0 hr	6 hr	24 hr
I	51.1 ± 6.8	53.9 ± 4.2	40.2 ± 16.4
II	51.5 ± 2.8	89.6 ± 4.3*	107.7 ± 12.9*
III	45.5 ± 5.7	50.5 ± 4.2^{NS}	55.9 ± 7.2^{NS}
IV	49.9 ± 7.1	65.2 ± 8.4$^{@}$	67.7 ± 34.7$^{@}$
V	54.4 ± 4.1	69.3 ± 13.4$^{\#}$	88.6 ±30.4$^{\#}$

Comparisons were made between: Group I vs II, III, IV, V, Values are expressed as mean ± SEM of 6 animals, Symbols represent statistical significance: * - P < 0.001, # - P < 0.01, @ - P < 0.05

Figure 2: HDL - cholesterol level in serum

mogen was read at 560nm. Enzyme activity (1 Unit) = 50 % inhibition /minute[7]. Glutathione peroxidase (GPx) was assayed by taking 200 µl of tris HCL buffer (0.4 M), 0.4 mM. EDTA along with 100 µl of sodium azide and 200 µl of enzyme preparation (hemolysate) and mixed well. Thereafter, 200 µl of reduced glutathione solution (2 mM) followed by 0.1ml H_2O_2 were added The overall reaction was arrested by adding 0.5 ml of 10% TCA. The precipitate was removed by centrifugation at 4000 rpm for 10 minutes. The absorbance was read at 12 nm using spectrophotometer. The non-enzymatic reaction rate was correspondingly assessed by replacing the enzyme sample by buffer. The results are expressed as nmoles /min /litre serum. GPx = GSH consumed / Min / Litre[8]. The method involved heating of biological samples with 0.8 ml saline, 0.5 ml of BHT and 3.5 ml TBA reagent for 11/2 min in a boiling water bath. After cooling, the solution was centrifuged at 2,000 rpm for 10 min and the precipitate obtained was removed. The absorbance of the supernatant was determined at 532 nm using spectrophotometer against a blank that contained all the reagents minus the biological sample. The values were expressed in nmol /dl serum[9].

Statistical analysis

The data were expressed as mean ± SEM. Results were analyzed statistically by one-way ANOVA followed by Dunnet's ?t' test. The difference was considered significant if p<0.001.

RESULTS

On preliminary phytochemical screening of methanol extracts of *Ziziphus jujuba* Mill showed presence of various phytochemical constituents. The methanolic extract showed the presence of alkaloids, phenols, flavonoids, saponins, glycosides, and terpenes. But carbohydrates, steroids, protein, tannins, gums and mucilage were absent.

Anti-hyperlipidemic study: Total Cholesterol The MEZJ treated animals at doses (125, 250, 500 mg) showed significant (P<0.05, P<0.01, P<0.001) protection against elevated cholesterol levels when compared to control group of animals. The standard drug treated group also showed significant (P<0.001) in-

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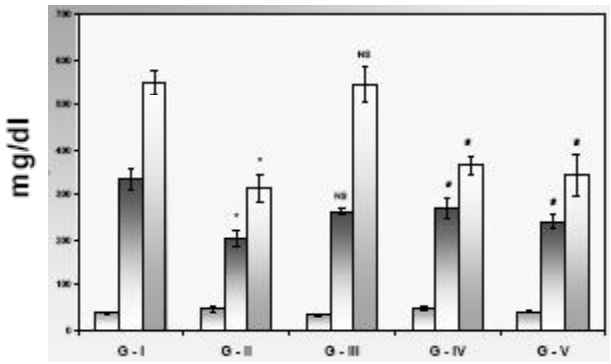
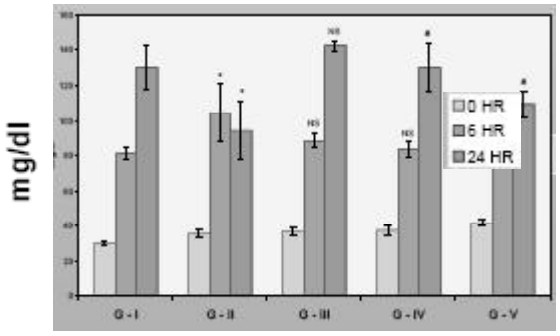


Group	LDL-Cholesterol (mg/dl)		
	0 hr	6 hr	24 hr
I	37.89 ±2.22	335.41±25.06	550.64±28.36
II	45.65±7.05	203.78±16.18*	314.03±29.13*
III	33.36±2.66	262.15±6.01^{NS}	547.25±40.45^{NS}
IV	47.89±4.49	270.28±21.77$^{\#}$	366.61±20.59$^{\#}$
V	40.22±2.65	239.52±16.46$^{\#}$	343.29±45.17$^{\#}$

Comparisons were made between: Group I vs II, III, IV, V, Values are expressed as mean ± SEM of 6 animals, Symbols represent statistical significance: * - P < 0.001, # - P < 0.01

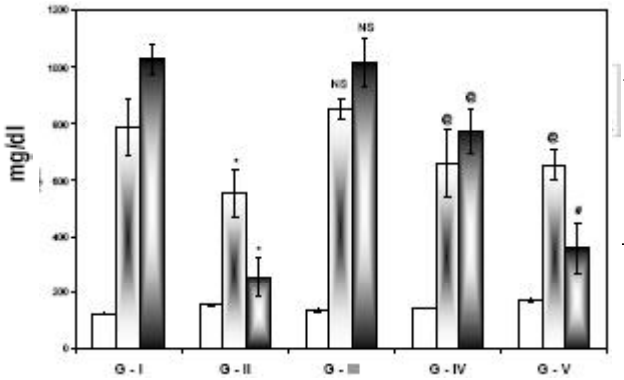
Figure 3 : LDL - cholesterol level in serum



Group	Glucose (mg/dl)		
	0 hr	6 hr	24 hr
I	30.13 ±1.6	81.44±3.2	129.91±12.4
II	35.54±2.6	97±6.7*	94.35±7.0*
III	37.14±2.4	88.19±3.9^{NS}	142.58±3.0^{NS}
IV	37.36±2.6	83.71±4.1^{NS}	130.21±8.3$^{\#}$
V	41.7±1.9	83.20±6.7$^{\#}$	109.3±7.7$^{\#}$

Comparisons were made between: Group I vs II, III, IV, V, Values are expressed as mean ± SEM of 6 animals, Symbols represent statistical significance: * - P < 0.001, # - P < 0.01

Figure 4 : Glucose level in serum



Group	Triglycerides (mg/dl)		
	0 hr	6 hr	24 hr
I	120.68 ±2.7	789.45±98.9	1027.25±38.8
II	151.48±2.8	554.17±87.0*	255.51±72.4*
III	133.75±6.5	851.17±33.6^{NS}	1017.12±86.6^{NS}
IV	141.41±2.7	657.77±120.4$^{@}$	774.71±80.5$^{@}$
V	169.98±6.1	655.51±56.2$^{@}$	357.77±86.4$^{\#}$

Comparisons were made between: Group I vs II, III, IV, V; Values are expressed as mean ± SEM of 6 animals; Symbols represent statistical significance: * - P < 0.001, # - P < 0.01, @ - P < 0.05

Figure 5 : Triglycerides level in serum

crease in cholesterol levels when compared to control. (Figure 1)

HDL

The MEZJ treated animals at doses (250, 500 mg) showed significant (P<0.05, P<0.01) protection in HDL levels against the increase in triton treated rats. The standard drug treated group also showed significant (P<0.001) protection in HDL levels when compared to control. (Figure 2)

LDL

The MEZJ treated animals at doses (250, 500 mg) showed significant (P<0.05, P<0.01) protection in LDL levels when compared to control. The standard drug treated group also showed significant (P<0.001) reduction in LDL levels when compared to control. (Figure 3)

Total glucose

The MEZJ treated animals at doses (250, 500 mg) showed significant (P<0.05, P<0.01) changes in glucose levels when compared to control. The standard drug treated group also showed no significant changes in glucose levels when compared to control. (figure 4)

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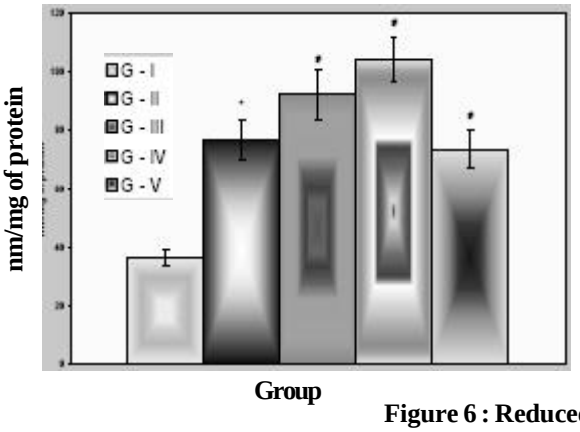
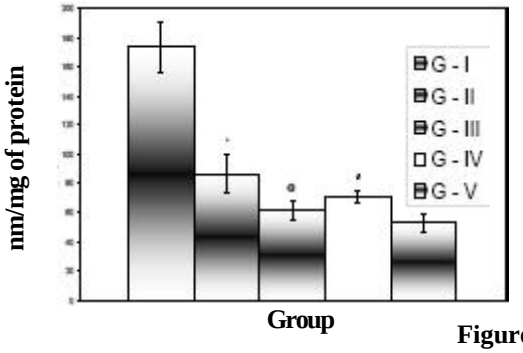


Group	I	II	III	IV	V
GSH (nm/mg of protein)	36.3± 2.9	76.7± 6.9*	92.58± 8.5$^{\#}$	104.42±7.7$^{\#}$	73.57± 6.4$^{\#}$

Comparisons were made between: Group I vs II, III, IV, V; Values are expressed as mean ± SEM of 6 animals; Symbols represent statistical significance: $^{\#}$ - P < 0.01, $^{@}$ - P < 0.05

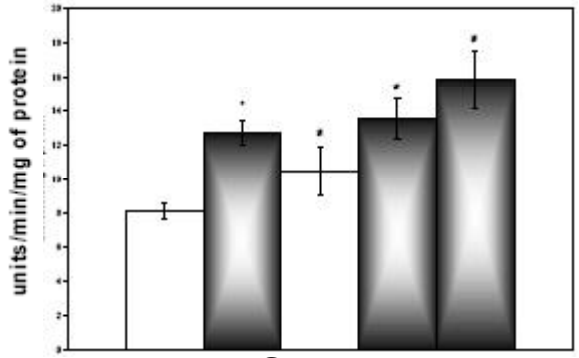
Figure 6 : Reduced glutathione levels in liver



Group	I	II	III	IV	V
Nitrite/nitrate (ng/mg of protein)	173.34 ±17.0	86.51± 13.1*	61.79± 7.1$^{@}$	71.5 ± 4.0$^{\#}$	52.87 ± 6.1$^{\#}$

Comparisons were made between: Group I vs II, III, IV, V; Values are expressed as mean ± SEM of 6 animals; Symbols represent statistical significance: * - P < 0.001, $^{\#}$ - P < 0.01, $^{@}$ - P < 0.05

Figure 7 : Nitate content in liver



Group	SOD(units/min/mg of protein)
I	8.10 ± 0.5
II	12.72 ±0.7*
III	10.50± 1.4$^{\#}$
IV	13.30 ± 1.2$^{\#}$
V	15.85 ± 1.7$^{\#}$

Comparisons were made between: Group I vs II, III, IV, V; Values are expressed as mean ± SEM of 6 animals; Symbols represent statistical significance: * - P < 0.001, $^{\#}$ - P < 0.01

Figure 8 : Superoxide dismutase levels in liver

Triglycerides

The MEZJ treated animals at doses (250, 500 mg) showed significant (P<0.05, P<0.05) protection in cholesterol levels when compared to control. The standard drug treated group also showed significant (P<0.001) reduction in cholesterol levels when compared to control. (Figure 5)

Reduced glutathione

The MEZJ treated animals at doses (125, 250, 500 mg) showed significant (P<0.01) protection in glutathione levels when compared to control. The standard drug treated group also showed significant (P<0.001) protection in glutathione levels when compared to control. (Figure 6)

Nitrate

The MEZJ treated animals at doses (125, 250, 500 mg) showed significant (P<0.05, P<0.01, P<0.01) protection in nitrate levels when compared to control. The standard drug treated group also showed significant (P<0.001) protection in nitrate levels when compared to control. (Figure 7)

Group	GPX (μm/min/mg of protein)
I	0.02 ± 0.005
II	0.08 ± 0.008*
III	0.01± 0.008^{NS}
IV	0.04 ± 0.007$^{\#}$
V	0.09 ± 0.013$^{\#}$

Comparisons were made between: Group I vs II, III, IV, V; Values are expressed as mean ± SEM of 6 animals; Symbols represent statistical significance: *-P<0.001, # - P < 0.01, N.S

Figure 9 : Glutathione peroxidase levels in liver

Group	TBARS (ng/mg of protein)
I	202.07 ± 19.6
II	143.51 ± 14.2*
III	207.20± 10.9$^{\#}$
IV	168.87 ± 18.7$^{\#}$
V	110.97 ± 15.7$^{\#}$

Comparisons were made between: Group I vs II, III, IV, V; Values are expressed as mean ± SEM of 6 animals; Symbols represent statistical significance: * - P < 0.001, # - P < 0.01

Figure 10 : TBARS levels in liver

Superoxide dismutase (SOD)

The MEZJ treated animals at doses (125, 250, 500 mg) showed significant (P<0.01) increase in SOD levels when compared to control. The standard drug treated group also showed significant (P<0.001) protection in SOD levels when compared to control. (Figure 8)

Glutathione peroxidase

The MEZJ treated animals at doses (250, 500 mg) showed significant (P<0.01) increase in SOD levels when compared to control. The standard drug treated group also showed significant (P<0.001) protection in SOD levels when compared to control. (Figure 9)

TBARS

The MEZJ treated animals at doses (125, 250, 500 mg) showed significant (P<0.01) protection in TBARS levels when compared to control. The standard drug treated group also showed significant (P<0.001) protection in TBARS levels when compared to control. (Figure10)

DISCUSSION

Scientific evaluation of The plant *Ziziphus jujuba* Mill using standard experimental animal model revealed the antihyperlipidemic nature of the plant *Ziziphus jujuba* Mill leaf extracts against triton induced hyperlipidemia in rats.Triton WR-1339 is one of the many well known non-ionic detergents that induce the elevation of plasma cholesterol and triglyceride levels by increasing the hepatic cholesterol biosynthesis. It also increases the HMG-CoA reductase activity in the liver. The Triton WR-1339 model has therefore been examined not only as screening methods for hypolipidemic agents, but also as a means for elucidating lipid metabolism[10]. In this study the when the triton is administered the lipid levels (total cholesterol, triglyceride and LDL) in all the rats were elevated. The increased lipid levels in the rats plasma were expressed as hyperlipidemic rats in this study. The MEJG significantly decreased the levels of total cholesterol, triglycerides and LDL in the Triton induced hyperlipidemic rats in a dose dependant manner[11]. The level of HDL is generally inversely correlated with the risk of cardiovascular disease in both human and laboratory animal studies, and increased HDL decrease the development of atherosclerosis. Thus, an increase in the HDL level by supplementation with the MEZJ may be responsible, in part, for the anti-atherosclerotic effect in animals.

Since a great part of the cholesterol in the body is

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synthesized *de novo,* mostly in the liver, the search for drugs to inhibit cholesterol biosynthesis has long been pursed as a means to lower the levels of plasma cholesterol. As high levels of cholesterol are correlated with an increased occurrence of atherosclerosis the therapy for hypercholesterolemia is focused mainly on inhibition of the selective cholesterol biosynthesis pathway in the liver. The enzyme, HMG-CoA reductase, catalyzes the rate-limiting reaction on the mevalonate pathway and acts early on this biosynthetic pathway. Inhibitors of cholesterol biosynthesis, for instance, particularly those that inhibit HMG-CoA reductase, are widely used to lower the cholesterol level. The effectiveness of HMG-CoA reductase inhibitors to decrease plasma cholesterol levels in animals and humans is well documented[12]. Cholestyramine increases the production of bile acid from the ileum and inhibit its passive absorption in the colon, resulting in the stimulation of HMG-CoA reductase activity. In this present study, administration with the MEZJ significantly inhibited the HMG-CoA reductase enzymes activities toward cholesterol biosynthesis in cholestryramine-fed mice. Our results also show that the HMG-CoA reductase activity was increased in Triton WR-1339 induced hyperlipidemic rats, whish is in agreement with reports of other studies made[13]. However, such increased activities of hepatic HMG-CoA reductase were decreased after administration of MEZJ in dose dependent manner. Based on these results, it is proposed that MEZJ administration involved suppression of endogenous cholesterol biosynthesis by inhibiting the activities of hepatic HMG-CoA reductase.The possibility exists that the MEZJ might have unregulated the hepatic LDL receptor. Thus increasing the cholesterol clearance, In which case the mechanism still remains to be elucidated through further studies. It is possible, that in addition to up regulation of the hepatic LDL receptor, the suppression of endogenous cholesterol biosynthesis by the MEZJ limited the availability of cholesterol to the liver for VLDL production[14].

On the other hand, the administration of the MEZJ suppressed the increased TBARS level in hyperlipidemic rats. As hypercholesterolemia leads to the increased production of oxygen free radicals. It exerts its cytotoxic effect by causing lipid peroxidation, resulting in the formation of malondialdehyde (MDA). Determining lipid peroxidation via the TBARS level provides an indirect measurement of antioxidative deficit. A decrease in lipid peroxidation leads to a reduction in the arterial wall cholesterol content. Thus, the reduction of lipid peroxidation is associated with a decrease in the arthrosclerosis caused by hyperlipidemia[15].

It seems from the results that the MEZJ had an antioxidative effect and counteractive effect against hyperlipidemia to some extent. Therefore, our results demonstrate that, in addition to lowering cholesterol, the MEZJ also helped in inhibiting lipid peroxidation and the oxidation of lipoprotein in hyperlipidemic rats.

The plasma NO concentration was decreased in the hyperlipidemic model. Endothelial-derived NO exerts vasodilatory, growth regulatory and anti-inflammatory effects, thus being an important regulator of cardiovascular homeostatsis. However, oxidatively modified LDL activities endothelial cell, leading to an alteration of the functional and structural integrity of the endothelial barrier, thus resulting in endothelial dysfunction, namely the production of oxidative stress during LDL oxidation. The inactivation of NO, and the inhibitor of NO synthesis by nitric oxide synthetase (NOS), The reduced level of NO production in the hyperlipidemic rats was gradually increased by pretreatment with the MEZJ[16]. This may have been due to enhancing the detoxicating enzymes by supplementating the MEJZ in the liver and its antioxidant effect. Indeed treatment with the MEJZ resulted in an increase in the activities of CAT and GPx. SOD plays a important role in protecting the cells from oxidative damage by converting superoxide radicals into hydrogen peroxide. The resulting hydrogen peroxide is further metabolized by CAT and GPx, where by CAT detoxifies hydrogen peroxide and GPx catalyses the destruction of hydrogen peroxide and lipid peroxide[17].

Therefore the increased enzyme activities resulting from the treatment with MEJZ may prevent the oxidative damage by detoxification reactive oxygen species and inhibiting lipid peroxidation thus reducing hyperlipidemia. Further studies are required to isolate the active principle present in *Ziziphus jujuba* Mill and evaluate its pharmacological activity, which may give a potential hyperlipidemic drug.

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