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Hypolipidemic effect of ethanolic extract from *Pandanus amaryllifolius* leaves on triton WR-1339-induced hyperlipidemia in mice

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Abstract

The 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase is the key enzyme of cholesterol biosynthesis pathway in the liver. The present work aimed at investigating the hypolipidemic effect of total extract from *Pandanus amaryllifolius* Roxb. (PA) leaves through the inhibition of HMG-CoA reductase activity. PA extract showed a moderate *in vitro* inhibitory effect on activity of HMG-CoA reductase with IC_{50} value at range of 126.42 µg/ml as compared to that of atorvastatin about 7.77 ng/ml. In a mouse model of hyperlipidemia induced by a single intravenous injection of triton WR-1339, oral administration of PA extract significantly reduced total cholesterol as well as triglyceride levels as compared to hyperlipidemic mice. Furthermore, PA extract also inhibited HMG-CoA reductase activity with reduction of 32%, which were comparable to those of positive control, atorvastatin. In conclusion, PA extract clearly showed promising hypolipidemic effect by inhibiting HMG-CoA reductase activity.

Keywords: Hyperlipidemia, triton WR-1339, *Pandanus amaryllifolius*, HMG-CoA reductase.

1. Introduction

Nowadays, dyslipidemia is a well-known risk factor for the development of atherosclerosis, coronary heart disease, heart attack and stroke - the leading causes of death worldwide [1]. The major objective of treatment in dyslipidemia patients is to decrease LDL-C in order to reduce the risk of developing cardiovascular disease or peripheral vascular disease [2]. Therapies used in treatment and management of dyslipidemia include dietary changes, risk factors controlling and use of hypolipidemic agent.

The 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA) reductase (EC 1.1.1.34) is the key enzyme of cholesterol biosynthesis pathway in the liver, catalyzes the conversion of HMG-CoA to mevalonic acid [3]. Thus, one of the most effective ways reducing lipid levels is to control the synthesis of endogenous cholesterol by inhibiting HMG-CoA reductase enzyme [4]. In this way, statins plays an important role in management of dyslipidemia. Nonetheless, the medication of these drugs presents certain limitations and significant side effects on skeletal muscle as well as the liver [5]. Therefore, naturally derived therapeutic agents are being concerned in the treatment of dyslipidemia.

Pandanus amaryllifolius Roxb. (PA), belonging to Pandanaceae family, is used in traditional medicine to treat hyperglycemia in diabetic patients. Currently, many studies worldwide have elucidated this effect [6],[7]. In addition, PA leaf extract also exhibited strong antioxidant effect [8], selective inhibition of MDA-MB-231 tumor cell proliferation [9], antibacterial and antiviral activities [10],[11],[12]. However, there is a little study related to its hypolipidemic effect.

The present study aimed at investigating the hypolipidemic effect of total extract from PA leaves through the inhibition of HMG-CoA reductase activity *in vitro* and *in vivo*.

2. Materials and methods

2.1. Chemicals

Triton WR-1339 (T0307), atorvastatin (PZ0001), NADPH (N5130), HMG-CoA (H6132), dithiothreitol (D0632) were purchased from Sigma–Aldrich (GmbH, Germany). Total cholesterol, triglycerides, HDL-cholesterol and total protein assay kits were obtained from Centronics GmbH (Germany).

2.2. Animals

Male *Swiss albino* mice (25-35 g) were purchased from The Institute of Vaccines and Medical Biologicals Nha Trang, Viet Nam. They were acclimatized to a 12-h light-dark cycle for at least 2 weeks before each experiment. Mice were fed standard lab chow and water *ad libitum*. The mice were deprived of food in the night before each experiment. All experimental protocols were conducted under agreement of the scientific committee, specialty of Pharmacology and Clinical pharmacy, Faculty of Pharmacy, University of Medicine and Pharmacy at Ho Chi Minh city, Viet Nam (Number 03-2016/QD-SDHD).

2.3. Preparation of the extract

PA plants were harvested in Thu Duc district, Ho Chi Minh City, Viet Nam, between August and September 2016. PA leaves were washed, dried and ground to crude powder. The powder was then extracted by percolation method with ethanol 50%, followed by solvent evaporation at 70^0C under reduced pressure. By using this procedure, the yield of extraction was 21.47 % estimated from the starting dry weight of the leaves. The obtained extract was stored in air-tight container until further use.

2.4. Preliminary phytochemical analysis

PA crude powder was sequentially extracted by different solvent having ascending polarization: ethyl ether, ethanol and water. The phytochemical composition was determined in each extract by specific chemical reactions for the presence of fats and essential oil, carotenoids, triterpenoids, alkaloids, saponins, flavonoids, tannins, carbohydrates, alkaloids, cardiac glycosids, polyuronic compounds…

2.5. Preparation of liver microsomes

Mice were euthanized by CO_2 dried-ice and liver was rapidly separated. After washing well with saline solution, it was blotted, weighted and put into iced-cold 0.25 M sucrose solution. Each liver was cut into pieces; and wet tissue was homogenized by using a warring blender. The homogenate was centrifuged at 5,000 g for 15 min at 0^0C and the pellets were discarded.

The supernatant was then collected; 0.1 ml of 88 mM $CaCl_2$ solution per ml of supernatant was added. The mixture was placed on ice for 5 minutes with occasional shaking. After centrifuging at 0 ^{0}C for 35 minutes at 13,500 g, pellets were homogenized in 2 ml of enzyme preservative solution (containing 0.1 M Tris. HCl buffer at pH 7.4, 0.25 M sucrose solution and 1 mM dithiothreitol) at 0-4^{0}C. The homogeneous protein suspension was alliquoted and stored -20 °C until use [17].

2.6. Total protein quantification

Total protein in liver protein homogenates were determined by colorimetric method based on biuret reaction.

2.7. Enzyme HMG-CoA reductase activity assay

The determination of HMG-CoA reductase activity was performed as procedure described by Patel H. D. *et al.* with some modifications [17]. Total reaction volume was 1 ml and reaction was carried out at 37 ^{0}C. Quantitative buffer (containing 0.1 M Tris. HCl buffer, 1 mM EDTA and 75 mM NaCl) was mixed with 1 mM dithiothreitol, 0.1 mM NADPH and 60µM HMG-CoA. After pre-incubating for 5 min at 37^0C, 1 mg liver protein suspension was added (except blank sample). The kinetic absorbance was then read continuously against a blank sample at 340 nm in each 30 seconds interval for 5 minutes at 37^0C. The enzyme activity is calculated by the formula (1):

Activity (nmol/min/mgP)

$$= \frac{\left(\frac{\triangle OD\ sample}{min} - \frac{\triangle OD\ blank}{min}\right) \times TV \times n\ \times 1000}{12{,}44 \times V \times C \times LP} \qquad (1)$$

2.8. *In vitro* inhibitory effects of HMG-CoA reductase

Protein suspensions isolated from livers of healthy mice were used. The reference drug (atorvastatin PZ0001) was dissolved in DMSO. Studied extract was dissolved in quantitative buffer. For test, drug or extract were pre-incubated with for 5 min at 37^0C with 0.1 M Tris. HCl buffer, 1 mM EDTA and 75 mM NaCl, 1 mM dithiothreitol, 0.1 mM NADPH and 60µM HMG-CoA. Then, 1 mg liver protein suspension was added and mixed. The kinetic absorbance was then recorded for 5 minutes at 37 ^{0}C[17].

The % inhibitory activity is calculated by the formula (2):

$$\%\ \text{inhibition} = \frac{\text{Activity control} - \text{Activity test}}{\text{Acitivity control}}\ x\ 100 \qquad (2)$$

Based on % inhibition values, the regression equation was drawn and IC_{50} values of atorvastatin and PA extract were estimated at concentration producing 50% inhibition of enzyme activity

2.9. Hypolipidemic effect against Triton WR-1339 induced hyperlipidemia

Hyperlipidemia was induced by a single intravenous injection of triton WR-1339 at the dose of 250 mg/kg. In treated groups, mice were orally administered by gavage with reference drug atorvastatin (64 mg/kg) or PA extract (equivalent to 25 g dried powder/kg b.w.) whereas mice in hyperlipidemic group had no treatment after Triton injection. On the other hand, a sham group received a single intravenous injection of saline solution was also included.

Thus, animals were divided into the following four groups: Sham group (n=6) administered NaCl 0.9%; Hyperlipidemic group(n=7) administered triton WR-1339 (250 mg/kg); Atorvastatin group (=7) administered triton WR-1339 (250 mg/kg) and treated with atorvastatin (64 mg/kg); Test group (n=10) administered triton WR-1339 (250 mg/kg) and treated with PA extract (25 g/kg).

Twenty-four hours after Triton injection, animals were sacrificed. Cardiac blood was collected and used for the estimation of serum lipid profile by using enzymatic-colorimetric method. Liver samples were also harvested for the determination of HMG-CoA reductase activity [14],[17].

2.10. Statistical analysis

Data were collected and presented as mean ± standard errors of mean (SEM). The data was evaluated by Kruskal – Wallis test and Mann Whitney test using Minitab 17. Differences between groups were considered significant when $p < 0.05$.

3. Results

3.1. Phytochemical composition

The results of preliminary analysis of phytochemical composition showed that PA was rich essential oil, free triterpenoids, alkaloids, organic acids and reducing compounds (Table 1). In addition, carotenoids, flavonoids, anthocyanosides, proanthocyanidins, tannins and saponins were also presented.

Table 1: Phytochemical composition of PA

Compounds	Extracts			Results
	Ether	Ethanol	Water	
Fats	-			-
Carotenoids	+			+
Essential oil	++			++
Free triterpenoids	++			++
Alkaloids	+	+++	+	++
Coumarins	-	-		-
Antraglycosids	-			-
Flavonoids	+	+	+	+
Cardiac glycosids		-	-	-
Anthocynosids		+	+	+
Proanthocyanidins		+	+	+
Tannins		-	+	+
Saponins		+	+	+
Organic acids		++	++	++
Reducing compounds		++	++	++
Polyuronic compounds			-	-

3.2. *In vitro* inhibitory effect of PA extracts on HMG-CoA reductase

The linear relation between the percentage of inhibition enzyme and logarithmic concentrations of atorvastatin and PA extract are shown in Figure 1 and Figure 2, respectively.

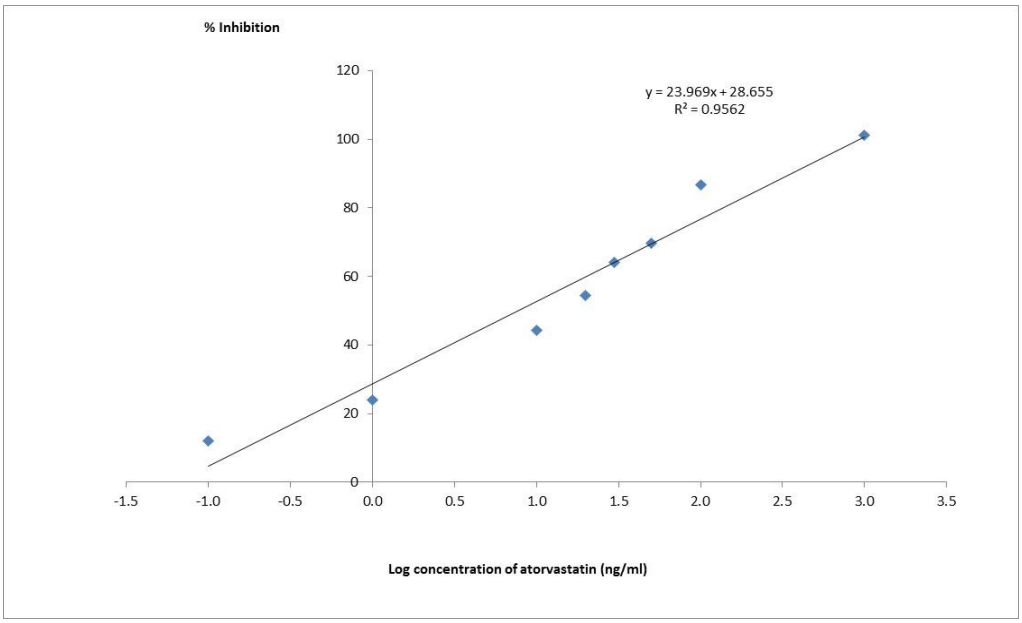

Figure 1: Effect of atorvastatin on HMG-CoA reductase activity

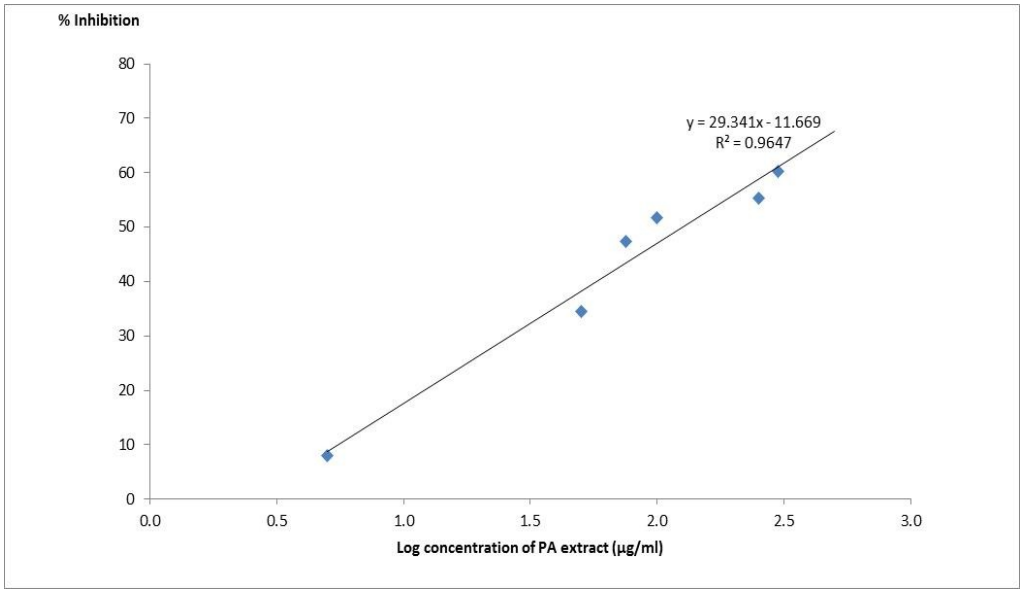

Figure 2: Effect of PA extract on HMG-CoA reductase activity

From the corresponding regression equations, the estimated IC_{50} of atorvastatin and PA extract were 7.77 ng/ml and 126.42 µg/ml, respectively.

3.3. *In vivo* inhibitory effect of PA extracts on HMG-CoA reductase

3.3.1. Lipid profile: The results of measurement of total cholesterol, triglycerides and HDL-c levels of mice were presented in Table 2.

Table 2: Effect of PA extract on total cholesterol, triglyceride, HDL-c levels and HMG-CoA reductase activity

Groups	Cholesterol (mg/dl)	Triglycerides (mg/dl)	HDL-c (mg/dl)	Activity (nmol/min)
Sham	**112.18 ± 8.06**	**137.58 ± 11.15**	**57.53 ± 1.46**	**6.27 ± 0.79**
Hyperlipidemia	305.61 ± 27.18*	1601.79 ± 238.85*	38.39 ± 1.7*	19.92 ± 1.47*
Atorvastatin	197.42 ± 22.24#	1071.13 ± 128.95#	41 ± 2.42	7.29 ± 4.18#
PA extract	215.61 ± 32.01#	920.77 ± 174.54#	44.09 ± 2.73	13.56 ± 1.81#

*p<0.05 vs Sham group, #p<0.05 vs hyperlipidemic group.

Twenty-four hours after triton WR-1339 injection, cholesterol and triglycerides levels significantly increased by 2.7 and 7.7 fold, respectively, in hyperlipidemic group as compared to the sham group. Moreover, HDL-cholesterol was also reduced by 1.5 fold and significantly different from sham groups.

The oral administration of atorvastatin at dose of 64 mg/kg significantly reduced total cholesterol (35%) and triglyceride levels (33%) compared to the hyperlipidemic group with a slight increase in HDL-cholesterol. Similarly, treatment with PA extract induced also a significant reduction in lipid levels as compared to the hyperlipidemic group. Indeed, total cholesterol decreased by 29% whereas triglycerides decreased by 42%. This reduction is almost comparable to that of atorvastatin. On the other hand, PA extract increased HDL-cholesterol (by 15%) but there was not significantly different from the hyperlipidemic group.

These results suggested the *in vivo* hypolipidemic effect of PA extract. Therefore, liver of experimental mice were isolated to determine the activity of HMG-CoA reductase enzyme from different groups.

3.3.2. HMG-CoA reductase activity

HMG-CoA reductase activity of mice groups are shown in Table 2.

After injecting triton WR-1339 for 24 hours, the HMG-CoA reductase activity of hyperlipidemic group increased 3.2 fold higher than and significantly different from that of the sham group.

Twenty-four hours after treatment with the reference drug atorvastatin at dose of 64 mg/kg or with PA extract at dose of 25 g dry powder/kg, the HMG-CoA reductase enzyme activity was significantly reduced as compared to the hyperlipidemic group (Table 2). Indeed, treatment with atorvastatin inhibited HMG-CoA reductase, leading to HMG-CoA reductase activity decreased by 63% compared with hyperlipidemic and returned to normal range as observed in sham mice. The oral administration of PA extract resulted in significant decline in HMG-CoA reductase activity with a reduction by 32% as compared to hyperlipidemic group. However, the enzyme activity of mice treated with PA extract was still higher than sham group as well as atorvastatin-treated group.

4. Discussion

Triton WR-1339 has been widely used in several previous studies in order to induce acute hyperlipidemia in animals [13],[14]. The increase of cholesterol and triglyceride in plasma is primarily caused due to an increase of VLDL excretion in the liver, concomitant of increasing strong catabolism of VLDL and LDL [13]. In addition, triton WR-1339 significantly increases the HMG-CoA reductase enzyme activity. In this study, HMG-CoA reductase enzyme activity mice injected triton WR-1339 increased 3.5 fold higher than sham mice. Moreover, the oral administration of atorvastatin at dose of 64 mg/kg immediately after injection significantly reduced serum total cholesterol and triglyceride levels but increased HDL-c levels and exhibited a significant inhibition of HMG-CoA reductase activity. This result showed the potential application of the hyperlipidemia model induced triton WR-1339 in order to tests evaluated lipid-lowering effects as well as HMG-CoA reductase inhibitory effects of various therapeutic agents. Several studies in the world have demonstrated that triton WR-1339 lowers cholesterol reserves in the liver, leading to increase HMG-CoA reductase activity and increasing cholesterol synthesis. The synthesis of cholesterol in the liver is maximized at 24 hours after triton WR-1339 injection [15],[16].

In *in vitro* studies at the HMG-CoA reductase inhibitory effect, homogenate from healthy mice liver was used. Experimentalresults showedthathomogeneous fluid from mice liver containing HMG-CoA reductase enzyme was ability to catalyze the conversion of HMG-CoA to mevalonic acid. Thus, this homogeneous fluid could be used to investigate the HMG-CoA reductase inhibitory effect of the active compounds or medicinal extracts. Indeed, the evaluation of *in vitro*HMG-CoA reductase activity after PA extract exposure at different concentrations showed that PA extract exhibited HMG-CoA reductase enzyme inhibitory effect, with an estimated IC_{50} about 126.42 µg/ml.

At a dose of 25 g dry medicinal powder/kg, the oral administration of PA extract has reduced lipid levels of experimental hyperlipidemic mice induced triton WR-1339 by lowering total cholesterol and triglyceride levels and by increasing HDL-cholesterol. In addition, this hypolipidemia effect should be due to the inhibition of HMG-CoA reductase enzyme. This is the first study demonstrating the lipidlowering effect and inhibitory activity towards HMG-CoA reductase of PA leaves. The effects of PA extract on HMG-CoA inhibition (13.56 nmol/min) is less than atorvastatin (7.29 nmol/min) but its cholesterol-lowering effects is quite similar to atorvastatin. This suggested that PA extract could lower cholesterol by many other mechanisms. According to several studies, flavonoids and saponins presented in medicinal extracts exhibited not only hypoglycemic but also lipid-lowering effects as well as the ability to inhibit HMG-CoA reductase enzyme [18]. Our preliminary phytochemical screening revealed the presence of these two components in PA leaves. In addition, PA leaves also possessedterpenoid compounds, which exhibited many benefits in the treatment of obesity, dyslipidemia, type 2 diabetes mellitus [19]. Hence, it can be suggestedthat the hypolipidemic of P.A might be correlated to these compounds. This research premised for further research on this plant to create a better healthcare products.

5. Conclusion

P. amaryllifolius extract clearly showed promising hypolipidemic effect by inhibiting HMG-CoA reductase activity.

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